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Transgenic Animals

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I. Introduction

In September 1980 Gordon *et al.* found that injection of genes into the pronucleus of the fertilized mouse egg led to integration and apparent retention of exogenous genetic material in all cells of the newborn animal.

This finding established a new experimental approach to mammalian development, the power of which can still not be fully estimated. Pronuclear microinjection has shed light on what were previously the most challenging problems in mammalian development and disease. Two other gene transfer systems, retroviral infection and embryonal stem (ES) cell transfer, rely on the same principle, and though less widely used, have contributed similar significant information.

So comprehensive has information from these animals been, that a thorough review of this field is rapidly becoming impossible. Transgenic mice have added immeasurably to knowledge of fundamentals of developmental genetics, immunobiology, neurobiology, oncology, and genetic disease. Despite these substantial contributions, all workers in this field would certainly agree that we have only begun to exploit the full power of this technology.

Because of the large number of transgenic animals now in existence, no effort is made here to list systematically every gene transfer experiment. Rather, the data will be referred to in the course of discussions of the biological principles addressed by the research. However, every effort has been made to recover all relevant publications from the literature.

A special acknowledgment is offered here to Elaine Diacumakos, recently deceased, who made important improvements in micromanipulation of mammalian cells (Diacumakos, 1973).

The development of multicellular eukaryotes from a single fertilized egg to the adult presents a most challenging, but also a most intriguing and exciting problem to the developmental geneticist. How do the cells of the embryo and fetus, all of which are clonal descendants of a single

progenitor, and all of which carry the same genetic information, interact and thereby regulate themselves such that differential gene function is established? As a corollary to this question, how are genes regulated in the developing organism so as to orchestrate properly the processes of cell determination, apportionment of cells to specific lineages, cell migration, and preprogrammed cell death? Underlying these questions are fundamental biological principles—principles whose understanding would have profound impact on our concept of development as well as the advance of genetic engineering technology.

The traditional approach to this problem entailed the tagging of cells so that their developmental fates could be followed. In the early part of this century, amphibian tissue primordia were grafted to specified regions of genetically distinct recipients. Then, the effect of the new environment on determination and differentiation of the graft was assessed (see. e.g., Spemann, 1918). Numerous ingenious experiments based on this approach were made possible because of the ability to rear amphibian embryos from fertilization to adulthood in the laboratory. However, such techniques could not be readily extended to mammals, where the progeny develop within the body of the mother. In addition, it was not—and in fact still is not—clear that all mechanisms of determination and differentiation in mammals are sufficiently well represented in classes of organisms such as amphibians that experimental models can be found within the latter group that accurately represent events of mammalian development.

A major step toward surmounting these difficulties was provided by the production of mouse aggregation chimeras. This technique entails removal of the zona pellucida followed by aggregation of genetically distinct cleavage-stage embryos. Subsequent culture of embryo aggregates leads to formation of single giant blastocysts (Tarkowski, 1961; Mintz, 1962), which, upon reimplantation into pseudopregnant females, develop into frank genetic mosaics (Mintz, 1965). In a sense, each donor embryo in the chimeric, or allophenic mouse was like the donor graft in amphibian experiments, but in the case of mice, donor cells were developmentally totipotent at the time of "grafting." Because of the large number of mutants and inbred strains with distinct genetic markers available, the allophenic mouse was effectively applied to a wide variety of problems including immune tolerance, susceptibility to malignancy, sex determination and function, and determination of the number of cells assigned to populate differentiated tissues of the adult (reviewed in Mintz, 1974). However, a number of features of the chimeric mouse system made it unsuitable for analysis of many important problems in development. Because of the complexity of cellular interactions characteristic of embryonic and fetal development, it was not always easy, when examining the adult chimeric

mice, to discern the developmental history of cell populations in the animals. Moreover, because each donor embryo in chimeras is actually a package of genes, these animals rarely provided insight into developmental regulation of specific loci.

Improved techniques for constructing chimeras, as well as the development of recombinant DNA and gene transfer technologies, created the potential for inserting single cloned genes into the germ lines of mice. Teratocarcinomas, malignancies derived from early embryo cells, were shown to become developmentally normalized after insertion into recipient blastocysts (Brinster, 1974; Mintz and Illmensee, 1975; Papaiannou *et al.*, 1975). Teratocarcinoma cells could be maintained in culture for prolonged periods, and still, after microinjection into the blastocoel, cooperate in the production of normally differentiated organs of chimeras (Papaiannou *et al.*, 1975).

The exploitation of this phenomenon for introduction of individual genes into mice was made possible by the advent of techniques for inserting purified DNA into cultured cells (Graham and Van der Eb, 1973). Uptake and expression of cloned genes by cultured cells after DNA-mediated gene transfer (DMGT), though a relatively rare event, could be detected by imposing selective regimes on cells that demanded foreign gene expression for cell survival (Wigler *et al.*, 1977; Maitland and McDougall, 1977; Bachetti and Graham, 1977). It thus became possible to insert foreign genes into teratocarcinoma cells by DMGT (Pellicer *et al.*, 1980), to introduce chromosomes by cell fusion (Illmensee *et al.*, 1978), or to select for genetic deficiencies in teratocarcinoma cells (Dewey *et al.*, 1977), and then to use the survivors for chimeric mouse production. This strategy proved difficult, however, when it was found that only in the rarest of instances would teratocarcinoma derivatives in chimeric mice contribute to the germ-cell population (Papaiannou *et al.*, 1978; Stewart and Mintz, 1981; Bradley *et al.*, 1984).

The frequency of gene transfer by standard DMGT was too low for direct exposure of embryos to exogenous DNA. However, the development of micromanipulation equipment (Diacumakos, 1973) made possible the direct injection of DNA into cells. This procedure was found to result in gene transfer with a high (5–20%) frequency (Graessmann *et al.*, 1979; Capecchi, 1980). The success of microinjection led Gordon *et al.* (1980) to explore the possibility of microinjecting molecularly cloned DNA directly into the early mouse embryo. The strategy involved microinjection of the pronucleus of the fertilized egg followed by oviductal implantation of surviving zygotes.

Of 78 newborns tested for incorporation, 3 were found to be carriers of the new gene (Gordon *et al.*, 1980). Restriction analysis and Southern

blotting demonstrated the presence of material specific for the microinjected sequence, which was a recombinant plasmid carrying the herpesvirus thymidine kinase *(tk)* gene and the simian virus 40 (SV40) early region. The gene transfer protocol, diagrammed in Fig. 1, stimulated many laboratories to initiate experiments with this system. In the following year Brinster *et al.* (1981), Costantini and Lacy (1981), E. F. Wagner *et al.* (1981), and Gordon and Ruddle (1981) confirmed the effectiveness of this methodology, and further demonstrated germ-line transmission of the donor genes (Costantini and Lacy, 1981; Brinster et al., 1981, Gordon and Ruddle, 1981). Moreover, Brinster *et al.* (1981) provided compelling evidence that foreign genes could be expressed in mice. Gordon and Ruddle (1983) offered the name "transgenic" to describe these animals, a term that has subsequently been applied to similar systems in a wide variety of organisms, both animal and plant. The protocol for producing transgenic mice has remained unchanged since first described, and has been by far the most commonly used technique for inserting genes into the mammalian germ line.

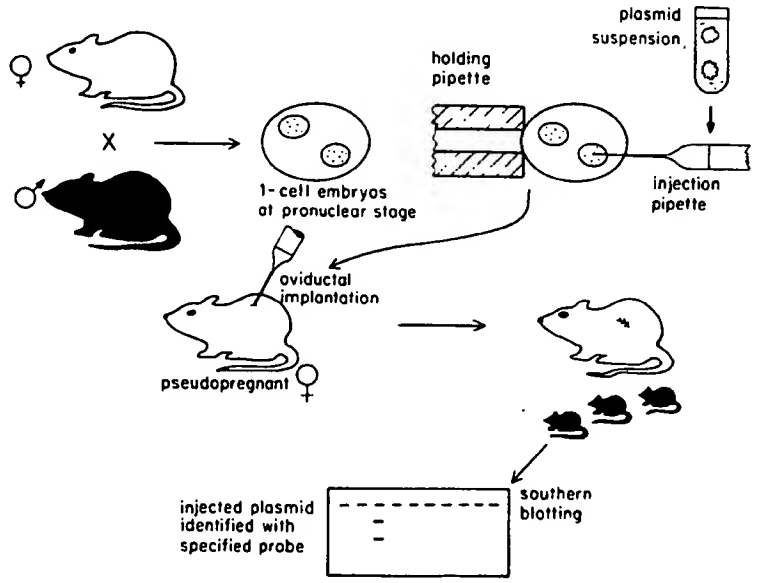

FIG. 1. Production of transgenic mice by pronuclear injection. One-celled fertilized eggs are recovered from females. A pronucleus is injected, and surviving embryos are reimplanted. (From Gordon *et al.*, 1980.)

In a separate line of research, Jaenisch and colleagues were investigating the potential for viruses to insert their genomes into early mouse embryos. Infection of mouse blastocysts with SV40 DNA resulted in persistence of SV40 sequences in adult animals (Jaenisch and Mintz, 1974; Jaenisch, 1974), although it did not appear that the viral genomes were integrated. However, infection of preimplantation mouse embryos with Moloney murine leukemia virus (MoMLV), an RNA retrovirus, led to insertion of a single copy of proviral DNA into the mouse genome, with transmission of the new material as a Mendelian trait (Jaenisch, 1976). Moreover, in at least some such strains of mice, the MoMLV genome was expressed, as evidenced by development of leukemia in the animals (Jaenisch, 1979).

The construction of retrovirus mutants defective in packaging but still able to produce proteins for packaging of heterologous recombinant retroviruses (Mann *et al.*, 1983) made possible the use of retroviral vectors for insertion of foreign genes into the mouse embryo (van der Putten *et al.*, 1985; Jahner *et al.*, 1985). This system has the drawbacks that the size of the DNA fragment that can be packaged in recombinant retroviruses is limited, and that expression of genes in such constructs is often poor (Jahner *et al.*, 1985). Later experiments have shown, however, that some internal promoters inserted into retroviruses function well and express their associated coding sequences in transgenic mice (Soriano *et al.*, 1986; Stewart *et al.*, 1987). Thus, the retroviral system can be useful for insertion of DNA into the germ line. The methodology is diagrammed in Fig. 2.

Concomitant with these developments in microinjection and retroviral infection of embryos, efforts continued to develop cell lines that could participate in formation of chimeric mice and also colonize the germ line. These efforts were important because, despite the many valuable experiments made possible by microinjection, the potential to manipulate cells in culture prior to insertion into animals offers unique advantages. Most important of these is the fact that selection can be applied to cells in culture. Not only can foreign gene transfer and expression be guaranteed by selection, but cells can also be selected for genetic deficiencies that, if transmissible through the germ line, would constitute models for human genetic disease.

Evans and Kaufman (1981) and Martin (1981) derived such cell lines from early embryos. These ES cells maintain karyotypic normalcy for prolonged periods in culture, and serve as recipients for gene transfer (Wagner *et al.*, 1985). Moreover, after gene transfer these cells, when reinserted into chimeras, can express foreign genes (Lovell-Badge *et al.*, 1985) and produce germ cells (Robertson *et al.*, 1986). These characteristics have recently been exploited to produce mice deficient in hypoxanthine phosphoribosyltransferase (HPRT), the enzyme deficiency responsible for

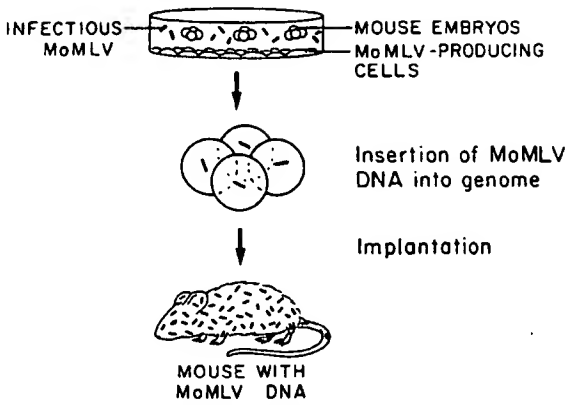

FIG. 2. Retrovirus-mediated gene transfer. Zona-free mouse embryos are cocultured with virus-producing stocks, or in medium containing retrovirus. After several hours of exposure, embryos are reimplanted. (From Jaenisch, 1976.)

Lesch–Nyhan disease in humans. ES cells selected for this deficiency by adding 6-thioguanine (6-TG) to culture medium (Hooper *et al.*, 1987), or by infecting cells with retroviruses and then selecting with 6-TG (Kuehn *et al.*, 1987), can transmit this deficiency through the germ line and yield colonies of animals with HPRT deficiency (see Section VI). The ES system still has drawbacks relative to microinjection or retroviral infection, because ES cells do not always populate the germ line, and the gene transfer protocol is more difficult. Nonetheless, the unique attributes of this system assure that it will assume an important role in future efforts to insert new genes into embryos. This methodology is depicted in Fig. 3.

In sum, there exist three viable strategies for insertion of genes into animals. By far the most productive of them has been microinjection. Although these technologies have only been available for a few years, their use has revolutionized our understanding of gene regulation in mammalian development. Not only have transgenic mice produced invaluable data relevant to mechanisms of tissue-specific gene expression, they have also allowed unprecedented studies of immune-system regulation and neoplastic disease, and have been applied to create models of human disease as well as treatment of diseases by gene therapy.

Most of these advances have relied on two important features of transgenic mice: the tissue-specific expression of donor genes and the ability of new DNA to interrupt the expression of endogenous genes by integration into the host genome. In this review I will first examine what is known

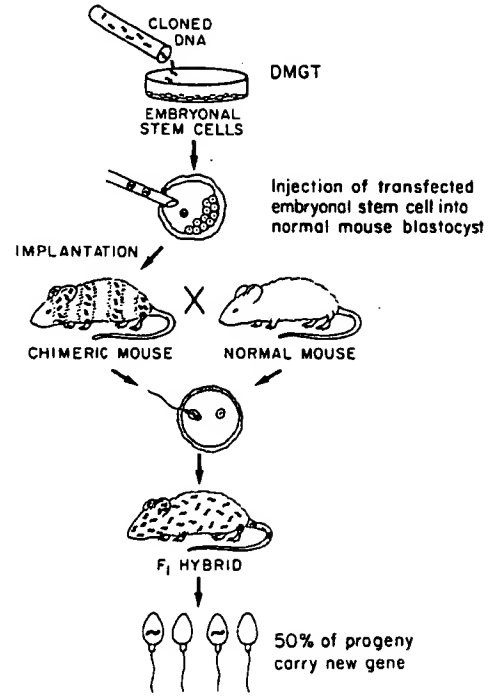

FIG. 3. Embryonal stem (ES) cell-mediated gene transfer. ES cells are transfected by a variety of means (in this case, DNA-mediated gene transfer, or DMGT), and transformed cells are injected into the blastocyst cavity. Chimeric mice that result are bred, and if sperm derived from ES cells contribute to the germ line, a transgenic pedigree is established.

about the mechanisms of foreign DNA integration in transgenic mice. Then, the fundamentals of gene regulation as revealed by transgenic mice will be discussed. The application of principles of gene regulation to the study of specific problems—including immune-system regulation, oncogenesis, creation of animal models for human disease, development of models for gene therapy, ablation of specific cell lineages in animals, studies of X-chromosome inactivation, and investigations of the effects of parental legacy on gene expression—will be described. Some of the unexplained observations on transgenic mice will also be mentioned. Then, the potential for creating mutations by foreign gene insertion will be reviewed. Finally, the prospects for future work in these areas will be outlined. Several other reviews, some comprehensive, some devoted to

specific areas of this field, have been or soon will be published (Gordon, 1983; Alt *et al.*, 1985; Gordon and Ruddle, 1985; Palmiter and Brinster, 1985, 1986; Scangos and Bieberich, 1987; Storb, 1987).

II. Foreign DNA Integration in Transgenic Mice

Relatively little attention had been focused on the mechanism by which exogenous DNA gains access to the mouse embryo genome. When DNA is transfected into cultured cells by calcium phosphate coprecipitation, unlinked markers become associated in large "transgenomes," which can remain unintegrated for many cell divisions (reviewed in Scangos and Ruddle, 1981). Independent donor molecules with sequence homology can also interact by homologous recombination en route to integration (Small and Scangos, 1983; DeSaint Vincent and Wahl, 1983; Shapira *et al.*, 1983). When microinjected into cultured cells, unlinked markers can also become linked and contransferred (Anderson *et al.*, 1980). Although homologous recombination between donor and chromosomal sequences can occur in culture, it is a rare event (Smithies *et al.*, 1985).

Restriction mapping of integrated transgenes in transgenic mice suggests that many of the mechanisms operative in cultured cells also exist in embryos. Although a formal investigation of the potential for cotransfer of unlinked markers has not been thoroughly conducted, donor molecules can become linked by homologous recombination prior to integration (Palmiter *et al.*, 1985b). In our own experiments we have observed cotransfer of independent markers that did not share sequence homology (J. W. Gordon and F. H. Ruddle, unpublished observations), and I am aware of several other laboratories that have made similar observations. Thus with regard to cotransfer of, and homologous recombination between, unlinked markers injected into embryos, mechanisms analogous to those present in cultured cells appear to exist.

Another similarity between genes inserted into cultured cells and embryos is that integration may be delayed. Delayed integration is manifest in mice as a mosaicism, detected by reduced frequency of foreign gene transmission through the germ line associated with an increase in hybridization intensity in transgenic progeny relative to the founder animal (Costantini and Lacy, 1981; Palmiter and Brinster, 1985; Wilkie *et al.*, 1986.). This is not dissimilar from events seen in culture, where transgenomes may persist as extrachromosomal units for many cell divisions prior to their ultimate integration (Pellicer *et al.*, 1978; Scangos *et al.*, 1981; Scangos and Ruddle, 1981).

Unlike tissue culture experiments, efforts to detect (Palmiter *et al.*, 1982a) or to facilitate (Brinster *et al.*, 1985) homologous recombination between exogenous and endogenous genes in mice have never been successful. However, the existence of a homologous recombination mechanism in embryos has not been ruled out. It is difficult to select for homologous recombination in transgenic mice, and most experiments with these animals have been focused on testing for gene expression rather than for homologous recombination. The ability to direct transfer of donor DNA by homologous recombination or other means is an important objective for future research.

A salient feature of integrated DNA in transgenic mice is the frequent insertion of large concatamers of donor molecules, usually as head-to-tail arrays (Costantini and Lacy, 1981). It is unclear how the concatamers form. Brinster *et al.* (1985) proposed that the limiting step to integration was breakage of host chromosomal DNA that allowed insertion of the linear microinjected molecule. Concatamers are then generated by integration of multiple additional molecules by homologous recombination with the first. While attractive, this hypothesis does not explain all characteristics of the integrated elements. For example, it is not uncommon to observe in such concatamers reduplication of restriction fragments not found in the original injected sequence. This suggests that the embryo is able to generate concatamers from one or a few donor fragments, because if concatamers always formed by homologous recombination between donor molecules, then each new added unit would be identical to the injected unit or a uniquely modified derivative of the original material. Although homologous recombination between donor molecules cannot be the only mechanism for generation of tandem arrays, such recombination may well occur. To my knowledge, no concatamer has ever been found in transgenic mice that does not contain at least one complete copy of the DNA fragment originally injected into the pronucleus. If concatamers were generated solely by reduplication of one fragment, and modification of that fragment occurred prior to concatamerization, then some mice should exist with concatamers composed exclusively of modified elements. That no such mouse has been found does indicate that at least some subunits are added by homologous recombination between donor molecules prior to integration.

It is probable that formation of concatamers is an intermediate step to integration, or at least increases the likelihood of integration. Burki and Ullrich (1982) and E. F. Wagner *et al.* (1981) have observed that while transgenic fetuses and their placentas may both have integrated tandem arrays of a transgene, the restriction maps of the integrated molecules may differ between placenta and fetus. This suggests that integration may be delayed and as a result, occur at different sites within the placental

and embryonic genomes (Burki and Ullrich, 1982; Wagner *et al.*, 1981). Because integration is a relatively rare event, it is unlikely for two entirely separate integration events to take place in the placenta and soma of a single embryo. Therefore, it is logical that formation of the concatamer, while not always followed immediately by integration, greatly increases the chance of integration. It is my own persuasion that once a concatamer forms, integration is inevitable. DNA inserted into mouse embryos by iontophoretic microinjection (Lo, 1983) appears not to result in concatamer formation. This technique has not yet been extensively applied to mouse embryo gene transfer.

The frequency of integration is highly variable. Evidence indicates that linearization of DNA prior to microinjection increases integration frequency (Palmiter *et al.*, 1982a; Brinster *et al.*, 1985), as does the strain of animals used and the number of copies of material inserted into the pronucleus (Brinster *et al.*, 1985). In our laboratory, microinjection of anywhere from 200 to 2000 copies of a gene into the pronucleus has no effect on the integration frequency. Insertion of high molecular weight DNA does not reduce the frequency of gene transfer (Palmiter *et al.*, 1982a).

Integration of microinjected genes may be associated with extensive rearrangements of the host genome. Analyses of integrated material or the pattern of foreign gene transmission have demonstrated that transgenes may be interspersed with "islands" of genomic DNA (Covarrubias *et al.*, 1986), some of which may originate from a distant site within the host genome. Integration has also been associated with deletions (Covarrubias *et al.*, 1986; Palmiter and Brinster, 1986; Scangos and Bieberich, 1987), duplications (Palmiter and Brinster, 1986), and translocations (Overbeek *et al.*, 1986; Scangos and Bieberich, 1987; Mahon *et al.*, 1988) within the host genome. It is unclear how frequently such anomalies occur, because most laboratories focus attention on transgene expression and do not formally study DNA structure in and around the integrated molecule. These events may have practical significance, however, when considering the use of gene transfer for genetic engineering of agricultural animals, because they are often associated with reduction in fertility or frequency of transmission of the new gene (reviewed in Scangos and Bieberich, 1987).

Even long after insertion, transgenes may be structurally unstable. Reduction in the size of the concatamer, probably by recombination between identical internal subunits, may lead to "looping out" of portions of the tandem array (Palmiter *et al.*, 1982a). One instance of transient amplification of exogenous material has also been reported (Shani, 1986).

A major advantage of retrovirus-mediated gene transfer is that host DNA rearrangements do not occur. The retrovirus inserts proviral DNA into the genome in a manner analogous to insertions that take place in the course of a typical cycle of infection: a single proviral copy of the genome is integrated (Jahner *et al.*, 1985; van der Putten *et al.*, 1985). Absence of concatamer formation is another attractive feature of the retrovirus system, because it facilitates molecular cloning of flanking DNA in instances where insertional mutations exist (see Section VIII).

The balance of current evidence indicates that integration occurs randomly within the host genome. As noted previously, efforts to detect recombination between endogenous DNA with sequence homology to donor genes have proved negative. In addition, *in situ*-hybridization experiments have failed to demonstrate integration of donor globin genes into chromosomal regions known to carry the mouse globins (Costantini and Lacy, 1981; Lacy *et al.*, 1983). New evidence suggests that retroviral DNA integrates preferentially into regions accessible to digestion with DNase I (Rohdewold *et al.*, 1987). These results were obtained by cloning DNA flanking retroviral inserts and using it as a probe for DNase I hypersensitivity in DNA from normal early embryos. No similar study has been conducted for animals produced by microinjection.

Two cases exist where the foreign DNA was clearly retained for extended periods in the absence of integration. Rassoulzadegan *et al.* (1986) microinjected a plasmid containing the polyoma large-T antigen, and reported that derivatives of the microinjected DNA were maintained for extended periods in the absence of integration. Evidence supporting this assertion included Southern blot analysis, a nearly 100% frequency of transmission of the foreign DNA, and the ability to rescue plasmids from transgenic mouse tissues. The rescued plasmids were highly modified relative to the original clone, in that polyoma sequences were highly modified or lost, and mouse DNA was acquired. It was suggested the mouse DNA contained sequences with centromeric function, which allowed for segregation of the unintegrated material (Rassoulzadegan *et al.*, 1986). The precise conditions that predisposed to these events are still not entirely clear (Leopold *et al.*, 1987).

The other case where unintegrated DNA was found involved microinjection of bovine papilloma virus (BPV) DNA. A dimer of the fragment of BPV comprising 69% of the viral genome and known to contain elements for viral replication was inserted along with a single copy of the remaining 31% of the virus. In this case, viral activation led to apparent excision of circular virions from the genome by recombination between the duplicated 69% regions (Lacey *et al.*, 1986). However, autonomous elements were not found in tissues in which the virus was silent and were not transmitted through the germ line with unusual frequency.

SUMMARY

Under ideal conditions, the frequency of gene transfer by microinjection is 15–30%. Genes may integrate singly or as head-to-tail concatamers, usually in a single site in the genome. Integration occurs shortly after microinjection, but may be delayed for a time sufficient to result in germline and somatic mosaicism. Unlinked markers may be cotransferred, and independent microinjected molecules may interact by homologous recombination en route to integration. For all practical purposes integration occurs randomly within the recipient genome.

III. Foreign Gene Expression in Transgenic Mice

Transgenic mice have contributed significantly to our understanding of differential gene expression. All of the applications of transgenic technology to studies of the immune system and of malignant disease, to the creation of animal models for human disease, and to the development of models for gene therapy and for the genetic manipulation of the phenotypes of animals, are based on these precepts.

Even before the first transgenic animals were produced, it was evident that three factors must determine how genes are regulated. First, cis-acting elements in and around genes must be present for initiation of transcription; second, trans-acting factors must be present in cells to stimulate gene activity; and third, the chromosomal site of integration must influence the ability of genes to turn on. What was unclear was the relative importance of the three elements in determining tissue specificity, developmental timing, and level of expression. For example, a system of gene control could readily be envisioned in which cis- and trans-acting factors were the same for all genes, and differential regulation depended on the integration site, which somehow was opened at the appropriate time and place when expression was required. Transgenic mice have provided a new perspective on the relative importance of cis- and trans-acting factors, and on integration site, to differential gene regulation. These three paramaters will be considered separately, after which their interaction to produce anomalous expression will be discussed.

A. Cis-Acting Elements

Early experiments with transgenic mice clearly demonstrated that, contrary to expectations, cis-acting elements in or around genes were highly important in determining tissue specificity of expression. These experiments involved introduction of gene constructs carrying the promoter region of mouse metallothionein-I (MT-I). MT-I is ubiquitously expressed, but is most active in liver and kidney, and its expression is inducible by heavy metals or corticosteroids. Brinster *et al.* (1981) linked this promoter (5′ upstream) region to the herpesvirus *tk* gene. Several lines of animals were produced, each with the chimeric gene integrated at a different site, and no evidence could be found for integration within the endogenous mouse MT-I gene (Palmiter *et al.*, 1982b). However, all lines expressed the *tk* gene preferentially in liver and kidney, with no expression detected in brain. Moreover, expression was inducible by injection of the heavy metal cadmium (Brinster *et al.*, 1981; Palmiter *et al.*, 1982b). These experiments indicated that cis-acting regulatory elements near or within the MT-I promoter were highly important to regulated expression. Such cis-acting sequences are often in the 5′ regions near the promoters of genes. In the case of elastase-I, 205 base pairs (bp) of DNA immediately 5′ to the ATG codon is sufficient to direct expression of heterologous genes to the exocrine pancreas (Ornitz *et al.*, 1985a,b, 1987; Quaife *et al.*, 1987). The 5′ end of the insulin gene similarly directs expression to the pancreatic islet cell (Hanahan, 1985). However, tissue-specific enhancers may also be located 3′ to genes (Kollias *et al.*, 1986; Storb, 1987; Behringer *et al.*, 1987; Trudel *et al.*, 1987), as far as 10 kilobases (kb) 5′ to genes (Pinkert *et al.*, 1987), or even within coding sequences (Stout *et al.*, 1985; Behringer *et al.*, 1987; Trudel *et al.*, 1987). Because enhancers have not been unequivocally identified within all promoter regions, cis-acting regulators will be interchangeably referred to as promoters, enhancers, or promoter–enhancer elements.

Strong confirmation of the importance of promoter–enhancer elements was provided by a number of experiments in which the MT-I 5′-flanking region was linked to heterologous genes, or in which small genomic clones of genes from various species were introduced into mice. When the MT-I promoter was fused to the rat (Palmiter *et al.*, 1982b) or human (Palmiter *et al.*, 1983a) growth hormone (GH) gene, substantial quantities of GH were produced from the livers of animals, and expression was induced further in many pedigrees by addition of zinc to drinking water. As a result of increased GH production, transgenic animals grew as much as two times faster than controls, and continuous, high ectopic expression of GH resulted in profound atrophy of pituitary acidophilic cells (Palmiter *et al.*, 1983a), as well as sex-specific alterations in hepatic function (Norstedt and Palmiter, 1984).

Intact genomic sequences were also shown to express appropriately in mice, even when derived from heterologous species. Immunoglobulin (Ig) light-chain genes expressed specifically in transgenic mouse B cells (Brinster *et al.*, 1983; Storb *et al.*, 1984), and the chicken transferrin gene

was expressed in mouse liver (McKnight *et al.*, 1983). Similar results have now been obtained for globin (Chada *et al.*, 1985; Townes *et al.*, 1985b), elastase-I (Swift *et al.*, 1984), myosin (Shani, 1985), albumin (Pinkert *et al.*, 1987), insulin (Bucchini *et al.*, 1986; Selden *et al.*, 1986), protamine (Peschon *et al.*, 1987), β-lactoglobulin (Simons *et al.*, 1987), superoxide dismutase (Epstein *et al.*, 1987), and other genes that will be discussed in more detail in later sections of this paper. These experiments indicate that cis-acting regulators, even if derived from evolutionarily distant species, can be recognized by mouse trans-acting factors. As will be discussed later, a number of factors including the integration site have a significant effect on gene expression. However, it is generally true that when expressed, even genes from heterologous species carrying their associated promoter–enhancer elements do so in a tissue-specific manner regardless of their integration site. Thus, it is a reasonable generalization from transgenic mouse data that it is not the chromosomal environment that regulates the availability of genes for transcription, but rather it is genes, via their promoter–enhancer elements, that regulate the chromosomal environment. A particularly elegant demonstration of this principle comes from experiments of Costantini *et al.* (1985), who studied the distribution of DNase I-hypersensitive sites in a hybrid mouse–human globin gene introduced into mice. Even though integrated randomly, this expressed transgene exhibited the same pattern of DNase I hypersensitivity (three sites evaluated) as the endogenous human gene in adult bone marrow.

1. *Variable Promoter Strength*

Cis-acting regulators vary in potency. Expression of globin genes is often low relative to the endogenous gene (Chada *et al.*, 1985; Townes *et al.*, 1985b), suggesting a relatively strong influence of the integration site. When compared with globin, Ig gene expression is consistently higher and less modified by integration site (Brinster *et al.*, 1983). When the rat pancreatic elastase gene was inserted, still another pattern of expression was seen. Not only was expression high and specific for pancreatic acinar cells, but the level of expression also roughly correlated with the number of copies of the gene present per cell of the transgenic mice (Swift *et al.*, 1984). This suggested that promoter–enhancer elements are graded in potency, with classes of genes such as globins having weak enhancers, immunoglobulins stronger ones, and genes such as the elastase the strongest. The weaker the promoter–enhancer element, the more obvious becomes the influence of the integration site on expression.

Experiments of Grosveld *et al.* (1987) indicate that variable promoter strength may not be due to intrinsic differences in cis-acting regulators of genes, but rather to the location of such regulators. Working with the adult β-globin gene, the cis-acting element of which had previously appeared weak, they grafted DNA normally located as far as 50 kb from the gene onto a recombinant plasmid in such a way as to approximate closely these DNA segments to the globin-coding sequence. The DNA segments chosen for transplantation to the globin gene contained sites highly sensitive to DNase I in the globin DNA of bone marrow, where the adult gene is normally expressed. When two such DNase I-hypersensitive sites were moved adjacent to the globin gene and the new construct was inserted into transgenic mice, the transgene adopted a pattern of expression typical of genes with the strongest promoter–enhancer elements: expression was influenced relatively little by integration site, and the number of copies of the transgene correlated directly with the level of expression. This is a highly significant finding, because it indicates that all genes may be associated with powerful enhancers, and that a systematic search for DNase I-hypersensitive sites near genes may lead to isolation of such enhancers. Similarly, Pinkert *et al.* (1987) have located an enhancer 10 kb 5′ to the albumin gene.

2. *Multiple Enhancer Elements*

Expression of some genes has been demonstrated to depend on multiple enhancer elements. The α-fetoprotein gene, which is expressed in visceral endoderm of the yolk sac, fetal liver, gastrointestinal (GI) tract, and regenerating liver, has been shown to be regulated by three 5′-enhancer elements (Hammer *et al.*, 1987). The Thy-1 gene, a cell surface antigen of the Ig gene family, is expressed throughout the brain, in the thymus, and in peripheral T cells of mice. When an 8-kb genomic clone of mouse Thy-1 was microinjected, it expressed in only a subset of appropriate sites. Expression in peripheral T cells and special regions of the brain did not occur (J. W. Gordon *et al.*, 1987). This indicated that multiple enchancer elements, some of which were not included on the microinjected clone, regulate the tissue distribution of expression of this gene. Widera *et al.* (1987) microinjected two fragments of the same major histocompatibility complex (MHC) gene into mice, one construct having 1.4 kb of 5′-flanking DNA and the other, 2 kb of 5′ flank. Both clones expressed in thymus and splenic T cells, but only the larger fragment expressed in B cells (Flavell *et al.*, 1986; Widera *et al.*, 1987). This again suggests the existence of multiple enhancer elements. A particularly interesting example of multiple regulators comes from the adult globin gene. Several workers have documented the existence of two 3′ enhancers, one in the third exon, the other 3′ to the polyadenylation site (Kollias *et al.*, 1986; Behringer *et al.*, 1987; Trudel *et al.*, 1987). Behringer *et al.* (1987) have shown that the 5′ region of the adult gene will not induce the fetal human gene to express

in the adult pattern; however, when the 3′ elements are present, this 5′ region is more active at directing adult-specific expression than the 3′ elements alone. Moreover, both 3′ enhancers work better than either one by itself. These findings indicate that enhancer elements can act cooperatively. In none of these experiments were the relatively distant DNase I-hypersensitive sites identified by Grosveld *et al.* (1987) included on the microinjected clones. These latter regions must also be considered stimulatory to tissue-specific globin expression, and thus, as many as five elements may be important for normal adult globin regulation.

The data can be summarized as indicating that multiple discrete regions in the vicinity of genes interact to induce changes in the organizational state of chromatin. These changes are tissue-specific and allow the gene to turn on.

3. *Vector Inhibition*

Promoter–enhancer elements of some genes are sensitive to perturbations of gene structure induced by the presence of DNA derived from cloning vectors. T. E. Wagner *et al.* (1981) initially reported that some transgenic mice carrying rabbit globin genes linked to plasmid DNA actively expressed the exogenous genes. However, subsequent studies clearly showed profound inhibition of globin gene expression by bacteriophage or plasmid sequences (Lacy *et al.*, 1983; Chada *et al.*, 1985; Humphries *et al.*, 1985; Townes *et al.*, 1985b). While globins are exquisitely sensitive to vector inhibition, other genes are only partially inhibited. The α-fetoprotein gene, normally expressed in yolk sac and fetal liver, is expressed in fetal liver in the presence of plasmid DNA, but yolk sac expression is extinguished (Krumlauf *et al.*, 1985c). For MHC genes expressed in spleen and thymus, plasmid DNA eliminates expression in spleen but not thymus (Widera *et al.*, 1987). In contrast, genes are not influenced by cloning vector-derived material. In a survey of 57 transgenic lines with Ig genes (Storb, 1987), 3 of 27 with vector sequences did not express, and 4 of 40 without vector were inactive.

These data indicate that the ability of some enhancers to open chromatin can be blocked by alteration of the surrounding DNA sequence. It is not clear at this time if, in cases of partial vector inhibition, some enhancers are blocked while others are not, or whether a single enhancer can be blocked in one tissue but not another. It is also unclear whether inclusion of all elements needed for enhancement could invariably eliminate vector inhibition. Globin genes previously noted to be inhibited did not carry the distant enhancer regions identified by Grosveld *et al.* (1987).

B. Trans-Acting Factors

Some specificity of gene expression is conferred by trans-acting factors. Because trans activators of gene expression are themselves products of genes, it is not possible to have a unique factor for every gene. Rather, single factors must be responsible for activation of several genes. If this is the case, then evolution of genes encoding trans-acting factors would be expected to proceed slowly, because mutations in such genes would, by virtue of their indirect effects on production of many proteins, be likely to have a significant disruptive effect on development.

This principle has been clearly demonstrated by transgenic mice carrying human Thy-1 genes. The Thy-1 protein is expressed in several specific tissues of the mouse, and in an overlapping but distinctly different set of tissues in humans. Thy-1 is made in the brain of both mouse and human, but is made in peripheral T cells only in mice, and in peripheral nerves only in human. When the human Thy-1 gene is introduced into mice, it assumes a pattern of expression indistinguishable from the human (J. W. Gordon *et al.*, 1987). This indicates that trans-acting factors for Thy-1 activation are present in mouse tissues that do not express Thy-1. The fact that these factors are still able to activate the human gene in a pattern identical to that found in humans indicates that the evolutionary divergence of Thy-1 expression results not from changes in trans-acting factors, but rather from qualitative changes in cis-acting elements in and around the Thy-1 gene (J. W. Gordon *et al.*, 1987). That trans-acting factors capable of activating Thy-1 are present in mouse tissues that do not express the mouse gene indicates that they serve other functions—undoubtedly, the activation of other genes.

Because embryonic and fetal cells are often no less specialized than those of the adult, they undoubtedly regulate their genes by similar interaction of promoter–enhancer elements and fetal trans-acting factors. Thus, expression of genes specific to the embryo or fetus is not conceptually different from tissue-specific expression in differentiated adult cells; the only difference is that the specificity is for cells that exist only at an earlier stage of development. This point is well demonstrated by insertion of genes such as α-fetoprotein, which is normally expressed in yolk sac and fetal liver and is silent in adult liver. The appropriate pattern of expression is adopted after the gene is introduced into transgenic mice (Krumlauf *et al.*, 1985b). Other genes or their promoter regions, such as α-crystallins (Overbeek *et al.*, 1986), α_2(I)-collagen (Khillan *et al.*, 1986), actin (Shani, 1986), myelin basic protein (Readhead *et al.*, 1987), and adult globin (Magram *et al.*, 1985), are similarly activated at the appropriate developmental stage.

However, although developmental regulation of genes after insertion into mice is often normal, timing of expression can change as a result of interspecies differences in the ontogeny of trans-acting factor production. The mouse adult β-globin gene is first expressed in fetal liver, and, postnatally, in bone marrow. When the human adult β-globin gene is inserted into mice, it follows a pattern analogous to the mouse adult gene (Chada *et al.*, 1985; Townes *et al.*, 1985b). However, when the human fetal gene is introduced into mice, it is expressed in yolk sac, a pattern characteristic of mouse embryonic globin (Chada *et al.*, 1986; Kollias *et al.*, 1986). This indicates that trans-acting factors capable of inducing expression of human fetal globin are present in mouse embryonic tissues. Hence the timing, and thus the tissue specificity of expression are determined by the ontogenic regulation of trans-acting factor production. Kollias *et al.* (1986) have further shown that a hybrid γ/β-globin gene, with the 5′ end derived from fetal and the 3′ end from adult globin, is expressed throughout gestation. Thus, a promoter–enhancer element(s) that makes human fetal globin accessible to transcription is in the 5′ end of the fetal gene, while an enhancer(s) of adult globin is apparently present in the 3′ end. The timed production of trans-acting factors in the developing mouse then induces expression of the hybrid gene at all stages of development.

C. Integration Site

As noted previously, integration site is relatively unimportant in determining tissue specficity of gene expression. However, the chromosomal domain is not entirely without significance. When human adult β-globin gene is inserted into animals, expression is tissue-specific in all expressing lines, but the level of expression varies markedly (Chada *et al.*, 1985; Townes *et al.*, 1985b). In addition, nearly every series of transgenic mice contains one or more pedigrees in which expression does not take place at all. This indicates that different chromosomal regions vary in their permissiveness for expression. Because some genes are affected relatively little by integration site (Brinster *et al.*, 1983), and because inclusion of DNase I-hypersensitive regions on microinjected globin genes can eliminate the sensitivity of these genes to potentially inhibitory effects (Grosveld *et al.*, 1987), it is unclear whether endogenous genes with all associated cis-acting sequences would ever be significantly dampened in expression by a change in chromosomal position. Such inhibitory influences may be particularly important for transferred genes, where the donor molecule may lack the full complement of enhancers found near their endogenous counterparts.

"Leaky expression" can also occur when transgenes integrate fortuitously in regions normally open for expression of endogenous genes. The first of numerous examples of such expression was reported for a rabbit globin gene, which exhibited low levels of expression in skeletal muscle of one line and testis of another (Lacy *et al.*, 1983). Integration of transgenes with strong promoters into highly permissive integration sites can broaden considerably the tissue distribution of significant expression. Stewart *et al.* (1984) produced a series of transgenic mice carrying the mouse mammary tumor virus (MMTV) promoter fused to the c-*myc* protooncogene. In most expressing lines animals developed breast tumors. However, in one pedigree tumors were found in a wide variety of sites, indicating that this integration site predisposed to a wider tissue distribution of expression. Similar results have been reported for expression of a rabbit β-globin gene driven by a retroviral promoter (Soriano *et al.*, 1986).

In sum, the integration site usually affects the level of transgene expression, while cis-acting elements determine the distribution of expression and, in conjunction with trans-acting factors, the developmental timing of expression. It remains possible that if all relevant cis-acting regulators were present on all donor genes, that integration site would never affect the level of expression. Ectopic expression can occur when genes integrate into highly permissive sites.

D. Anomalous Expression of Recombinant Constructs

Given the influences of the integration site, the presence of novel structures of recombinant transgenes that can act as novel enhancers, and the production within all cells of trans activators of gene expression, it should not be surprising that unusual patterns of expression are frequently seen. When novel, chimeric genes are introduced, two or more components of which carry their own cis activators of gene expression, how will the organism react? In some instances, one enhancer element may override all others and lead to predictable expression. This pattern has been seen for genes carrying both the SV40 early-region enhancer and the MT-1 promoter. When such constructs are linked to the SV40 T-antigen gene and inserted, transgenic mice develop tumors of the choroid plexus (Palmiter *et al.*, 1985a). The identical effect is observed when the oncogenic SV40 T-antigen gene is introduced with its own enhancer but without the MT-1 promoter (Brinster *et al.*, 1984). Thus, the SV40 enhancer appears to dominate and render the MT-1 promoter irrelevant. When the SV40 72-bp repeat, a strong enhancer, is deleted from the chimeric constructs, the MT-1 promoter takes over and transgenic mice develop hepatocellular carcinomas (Messing *et al.*, 1985). Similar results have been seen for an MT-1–GH fusion gene and a rabbit β-globin gene microinjected as part

of a single recombinant construct. When both genes were injected together, rabbit globin was expressed in a distribution characteristic of MT-1. When the MT-1–GH gene was excised, the isolated globin gene was expressed in erythroid tissue (Townes *et al.*, 1985a). HPRT (Stout *et al.*, 1985), gonadotrophin (Low *et al.*, 1986), and GH (Swanson *et al.*, 1985) genes can also override the effects of the MT-1 promoter.

While such "enhancer dominance" leads to understandable patterns of gene expression, other combinations of genetic elements on single recombinant molecules can lead to unpredictable transgene behavior. For example, the human hepatitis B surface antigen (HBsAg) gene inserted into mice with its own regulatory apparatus is expressed, as might be predicted, in liver (Babinet *et al.*, 1985). However, when this same gene was inserted with the MT-1 promoter, another promoter highly active in liver, the construct expressed in liver, but expression was even higher in stomach of one animal and heart of another (Chisari *et al.*, 1985). Similarly, presomatostatin fused to MT-1 is inappropriately expressed and posttranslationally processed in the anterior pituitary (Low *et al.*, 1985). When the rabbit β-globin gene is combined with the chicken conalbumin promoter, expression does not occur. When the B cell-specific Ig heavy-chain enhancer is added to the construct, expression occurs in B cells, but also in liver, testis, thymus, and brain (Gerlinger *et al.*, 1986). When the GH-releasing factor promoter, a promoter presumably specific for brain, is fused to the SV40 T antigen, which is also expressed in brain when linked to its own promoter (Brinster *et al.*, 1984), the new recombinant construct is expressed in the thymus (Botteri *et al.*, 1987).

Such anomalous expression probably occurs because novel constructs with multiple enhancer elements, or with such elements distributed in a way unlike any endogenous gene, can present spurious signals that allow new cell types to activate a transgene. These "false enhancers" are not confined to chimeric genes constructs. When the heavy chain of Ig, normally rearranged for expression only in the B-cell lineage, is microinjected in the rearranged form, it is expressed in T cells, heart, and brain (Grosschedl *et al.*, 1984; Nussenzweig *et al.*, 1987). Thus, simple alteration of the arrangement of sequences from a single gene can lead to novel expression.

An important consequence of the false-enhancer phenomenon is that it is difficult to design "reporter genes" that will test the tissue specificity of a given promoter–enhancer element in transgenic mice. An important potential application of the transgenic mouse is to link genes whose products can be readily detected, such as the bacterial chloramphenicol acetyltransferase (CAT) gene, to promoters whose tissue specificity is unknown, and to insert the hybrid genes into the mouse. By measuring CAT activity in various tissues, it would then be theoretically possible to define the tissue specificity of the promoter under study. When powerful, tissue-specific enhancers are used, such experiments can succeed (Khillan *et al.*, 1986; Overbeek *et al.*, 1986; Goring *et al.*, 1987). However, such novel constructs may well express in tissues in which the promoter, when linked to its native coding sequence, would be inactive.

E. Summary

Despite the aberrant features of foreign gene expression in transgenic mice, for all practical purposes recombinant molecules can be designed to direct expression of a gene to a tissue of choice. Usually the 5′ flanking region of a gene is employed as a targeting sequence, and is linked to a heterologous gene for microinjection. Although ectopic expression does occur, expression in the target tissue is usually far higher, and the physiological significance of targeted expression is so great that the experiment is not compromised. Thus, although abnormal gene expression in transgenic mice has been highly instructive regarding mechanisms of gene regulation, the tissue specificity of gene expression has had a far greater impact. The next sections will review the findings relating to tissue-specific gene expression, and the applications of transgenic mice to specific problems in mammalian development and disease.

IV. Transgenic Mice in the Study of Immunology

The immune-system genes are regulated by unique and intricate mechanisms. Immunoglobulin (Ig) molecules are composed of two identical heavy and light chains. The heavy-chain loci are encoded by three sets of subgenes: V_H, D_H, and J_H, while the light chains are encoded by V_L and J_L. Several remarkable features characterize Ig gene expression. First, the genes are physically rearranged for expression such that the functional gene differs substantially in structure from its corresponding locus present in all other somatic cells and in germ cells (reviewed in Tonegawa, 1983). Second, when one of the parental alleles is rearranged and expressed, the remaining allele is blocked from rearranging in a process known as allelic exclusion. Third, the genes are under tight developmental regulation. In the pre-B cell, μ heavy chains are synthesized; then light chains are made. Little is known about the switch from IgM to IgG production. It is not unequivocally demonstrated that γ-producing cells are descendants of IgM-secreting cells which secrete μ. With regard to light-chain synthesis, there also exists isotypic exclusion, a phenomenon that results in synthesis of

only one type of light chain, κ or λ. Finally there is somatic hypermutability of the *V–J* joining region. None of these idiosyncratic regulatory processes is well understood.

The immune system also encompasses the MHC genes. These Ig-like genes, inducible by interferon (IFN), are copresented with foreign antigens to T cells. They are thus required for antigen recognition.

Transgenic mice provide an unusually powerful probe into the various regulatory mechanisms of Ig genes. Genes have been cloned in their rearranged form and inserted into animals in order to study allelic exclusion, isotypic exclusion, and somatic hypermutability. In addition, cloned genes have been modified to block their ability to insert into the cell membrane or to be secreted, and the effects of such changes on allelic exclusion studied. Finally, the presence of a rearranged, expressible transgene in every cell, and expression of transgenes with production of functional immunoglobulins in some cells, can perturb the overall regulation of the immune system. Transgenic mice carrying Ig and MHC genes have thus provided profound insights into the genetic regulation of the immune response. In this section experiments, first with Ig genes, then MHC genes inserted into mice will be reviewed.

A. Ig Genes in Transgenic Mice

1. *Allelic Exclusion of Light-Chain Genes*

One of the first experiments involving Ig genes in transgenic mice was introduction of a rearranged, expressible κ light-chain gene cloned from a myeloma cell line MOPC-21 (Brinster *et al.*, 1983). The gene was actively expressed in several pedigrees (Brinster *et al.*, 1983), and expression was specific for B lymphocytes (Storb *et al.*, 1985). To study the effect of this functionally rearranged transgene on allelic exclusion of host genes, a series of hybridomas were produced from transgenic mouse B cells (Ritchie *et al.*, 1984).

Hybridomas exhibited a variety of patterns of transgene expression, each exerting its own effect on endogenous gene rearrangement and expression. In some instances, exogenous κ light chains were combined with heavy chains and secreted. When this occurred, endogenous κ gene rearrangement and expression was blocked. When transgene κ chains were present in the cytoplasm but not secreted, host gene expression, but not rearrangement, was blocked. Expression of endogenous light-chain genes without heavy-chain association and secretion did not block transgene expression. However, secretion of a mature Ig molecule derived entirely from endogenous genes was invariably correlated with the absence of transgene expression. The data indicate that expression of a rearranged

gene blocks rearrangement and expression of the other allele. However, if another allele is "pre-rearranged" as in transgenic mice, it is only blocked in expression by secretion of mature Ig with light chains derived from the other allele.

Reinforcing the fact that mature Ig molecules are required to exert allelic exclusion are two experiments wherein both μ and κ rearranged genes were inserted into mice (Rusconi and Kohler, 1985; Storb *et al.*, 1986). In one instance (Rusconi and Kohler, 1985) expression of the μ transgene vastly exceeded that of the κ gene, and endogenous κ rearrangement was not blocked. In the other case (Storb *et al.*, 1986), balanced amounts of the transgenes were made, and endogenous κ rearrangement was blocked. The requirement that heavy and light chains must be associated for blockage of light-chain rearrangement is interesting in light of the fact that μ-chain synthesis, even in transgenic mice carrying rearranged κ genes in the germ line (Storb *et al.*, 1985), precedes κ-chain synthesis. Because two rearranged κ genes are never expressed in a single B cell, this indicates that activation of one κ gene results in rapid saturation of μ chains with consequent allelic exclusion of the other light-chain locus (Storb, 1987).

2. *Allelic Exclusion of Heavy-Chain Genes*

Grosschedl *et al.* (1984) microinjected a functionally rearranged μ heavy-chain gene into embryos and evaluated four lines of resultant transgenic mice. The product of the transgene was specific for the hapten 4-hydroxy-3-nitrophenyl (NP). These mice produced antibodies to NP, although expression was variable between lines. A remarkable finding was that T cells expressed the rearranged μ gene, a finding confirmed in subsequent studies involving introduction of other rearranged μ transgenes (Storb *et al.*, 1986). Therefore, it appears that T cells are unable to express their μ genes because of their inability to rearrange these genes. This is interesting, because T cells rearrange their own Ig-like genes and clearly possess the enzyme systems required for rearrangement.

In addition to their aberrant expression in T cells, rearranged μ chains express in other sites. Grosschedl *et al.* (1984) detected low expression of heavy chains in heart, and Nussenzweig *et al.* (1987) detected expression in brain. In both cases contamination of these tissues by lymphoid cells was ruled out as the cause of expression.

To investigate the possibility of allelic exclusion exerted on endogenous μ genes by insertion of a rearranged transgene into the germ line, Weaver *et al.* (1985) established permanent cell lines derived from B-lymphocyte lineages in transgenic mice carrying the rearranged anti-NP μ gene of Grosschedl *et al.* (1984). Pre-B cells were immortalized by transformation with Abelson virus (AMLV), and lines derived from more mature B cells

were established by producing hybridomas from spleen cells. In contrast to normal controls, which showed no blockage of μ-gene rearrangement, 40% of AMLV-transformed cells from transgenic mice showed some blockage of endogenous μ rearrangement. Even in hybridomas established from transgenics, 10% of lines showed allelic exclusion of endogenous μ genes.

Further studies have demonstrated that it is the membrane-bound form of μ that causes allelic exclusion. Storb *et al.* (1986) injected μ genes in which sequences encoding the membrane terminus were deleted. In this instance, no allelic exclusion was observed. Correspondingly, Nussenzweig *et al.* (1987) inserted an altered μ gene whose product could not be secreted but could only be membrane bound. In this instance endogenous gene rearrangement was blocked.

Insertion of a rearranged human γ1 gene results in specific expression in spleen cells that was stimulated by lipopolysaccharide but not concanavalin A and that occurred in cells that stained positively for mouse IgM, results that indicate B cell-specific expression. However, a low level of T-cell expression could not be ruled out. Expression of endogenous mouse γ was not inhibited by the presence of the human transgene (Yammamura *et al.*, 1986), although transgene expression may have been too low to have significant effects on endogenous gene regulation.

3. *Isotypic Exclusion of Light-Chain Genes*

Insertion of rearranged, expressible light-chain genes into mice has also allowed investigation of isotypic exclusion. Normally, B cells express only κ or λ light chains, but not both. Under normal circumstances, productive rearrangement of κ genes is associated with blockage of λ rearrangement such that λ genes remain in the germ-line configuration. However, when λ genes are productively rearranged, κ genes are also either nonproductively rearranged or deleted. These observations have led to a "sequential" model for light-chain rearrangement, which states that B cells first attempt κ rearrangement. If a productive rearrangement occurs, λ genes remain in the germ-line configuration. If no productive rearrangements occur, the cell then proceeds to rearrange its λ genes (Hieter *et al.*, 1981).

If this model is correct, then insertion of expressed, rearranged κ genes into the germ line would be invariably associated with blockage of rearrangement and expression of λ chains. However, experiments showed this not to be the case. Some hybridomas established from transgenics with rearranged κ light-chain genes expressed both the transgene and a productively rearranged λ gene. In addition, nonproductive rearrangement and deletion of endogenous κ genes occurred despite the presence of complete Ig molecules derived from the exogenous κ chain (Storb, 1987). These observations led Storb (1987) to propose that separate B-cell lineages exist, κ and κ-λ. In the κ lineage only κ genes can rearrange, and λ genes remain in the germ-line configuration. In the κ-λ lineage, λ rearrangement can occur as well as κ, but rearrangement does not exert allelic exclusion upon κ genes. As a result, all κ genes continue to rearrange until all are either rearranged or deleted.

4. *Somatic Hypermutation*

In the joining regions of *V* genes, somatic mutations accumulate at a far higher rate than in other regions of the genome. Evidence that the rearrangement process results directly in the introduction of mutations consists of the finding that mutations are clustered at the rearrangement joining sites, that mutations are absent in unrearranged *V* genes, and that mutations occur at a relatively low frequency in partially *(D–J)* rearranged, nonproductive heavy-chain genes. Other evidence also exists, however, that these mutations accumulate following rearrangement. Data supporting this view stem from the observation that in independent hybridomas derived from a single mouse, several had some common mutations in the joining region. This suggested that the B cells contributing to the hybridomas were derived from the same precursor cell, and that the different patterns of mutations seen in the joining regions developed later, from mutations that occurred subsequent to rearrangement. In addition, the number of mutations increases with time after immunization. The latter data are consistent with the existence of a special "mutator" mechanism, which recognizes *V* joining regions and mutates them. However, the possibility that all mutations occur at the time of joining, and that those B-cell clones with the most extensively mutated genes have a proliferative advantage, was not ruled out as the explanation for increased somatic mutation with time after immunization.

Transgenic mice with rearranged genes inserted into the germ line allow a test of whether the rearrangement process is linked to hypermutation. O'Brien *et al.* (1987) hyperimmunized MOPC-21 κ transgenic mice, produced hybridomas, and selected for clones in which one of the endogenous heavy-chain *V* regions was mutated. Thus, clones were evaluated that were known to have undergone somatic mutation. When the κ transgenes, which had been rearranged prior to microinjection into the pronucleus, were evaluated, several *V*-region mutations were found, thus demonstrating that somatic hypermutation is not intrinsic to the process of gene rearrangement. Thus, the phenomenon of somatic hypermutation may result from a special mutator mechanism (Storb, 1987).

5. *Immune-System Perturbation by Ig Transgenes*

The presence of foreign, rearranged transgenes, particularly heavy-chain genes, can cause profound disturbances in differentiation and clonal proliferation of B cells. Storb *et al.* (1986) inserted a specific MOPC-167 κ and μ genes into transgenic mice. In mice that expressed the foreign μ heavy chain, a significant increase in endogenous MOPC-167 κ light-chain mRNA was detected in spleen. Similarly, mice expressing the MOPC-167 κ transgene showed an increase in endogenous MOPC-167 μ mRNA synthesis. These findings indicate that B cells that express a MOPC-167 transgene and also fortuitously express the corresponding endogenous gene, proliferate more rapidly and are preferentially represented in the spleen B-cell population (Storb *et al.*, 1986; Storb, 1987). Thus, the presence of the transgenes alters the pattern of B-cell proliferation. Interestingly, this clonal selection does not occur when the μ gene deleted for its membrane-binding moiety is inserted, even though the transgene is actively expressed. Apparently, stimulation of MOPC-167 clones in response to antigen or as an antiidiotype response requires the presence of membrane-bound Ig (Storb, 1987).

Marked abnormalities have been reported in mice carrying a rearranged anti-NP μ heavy-chain gene (Grosschedl *et al.*, 1984). Weaver *et al.* (1986) established hybridomas from these mice and observed that several clones produced anti-NP antibodies. However, the μ chain employed for antibody synthesis was apparently not derived from the transgene but from endogenous genes. The number of NP-reactive clones in a normal mouse is about 1%, but in these transgenics the number was 68% in lymph node-derived hybridomas and 28% in spleen hybridomas. Moreover, the V–D–J_H regions of the expressed genes were often structurally distinct from that of the transgene, indicating that antibody specificity did not result from integration of the transgene into the expressed locus. These data indicate that the transgene, though not itself utilized for antibody production, triggered the production NP antibodies or proliferation by clones expressing anti-NP antibodies from endogenous genes. An alternative explanation is that the transgene was in fact expressed, but that some nonproductive rearrangements of endogenous genes also took place.

Further studies of these animals showed marked alteration of the B-cell population. Herzenberg *et al.* (1987) studied the two major B-cell populations in these transgenic mice by fluorescence-activated cell sorting (FACS). One type of B cell is derived from bone marrow and can be identified in the FACS device by staining the surface with fluorescently labeled anti-IgD. The other population, Ly-1, does not express IgD. When B cells from the μ transgenic mice were stained with IgD and sorted, the IgD population was found to be missing entirely. The precursors of IgD-producing cells probably exist in the mice, as evidenced by the ability to transform pre-B cell clones with AMLV. However, these cells never appear to develop into IgD-producing cells. The finding apparently contradicts the report that numerous B cells are present that produce anti-NP antibodies from endogenous genes (Weaver *et al.*, 1986), because there is no obvious reason why such cells would not go on to express IgD. Although the mechanism remains ill-defined, it is clear that the presence of rearranged μ genes in the germ line results in elimination of a major B-cell subpopulation.

6. *Unrearranged Ig Genes in Transgenic Mice*

Goodhardt *et al.* (1987) inserted an unrearranged rabbit κ-chain gene consisting of a single *V* region, five *J* segments, and the κ *C* region into transgenic mice. In 11 of 11 lines tested, the transgene was rearranged. Only the Jk1 and Jk2 segments, which have canonical sequences for rearrangement, were utilized in assembly of the rabbit gene. Remarkably, Vk–Jk1 and VK–Jk2 joining was also observed in thymus, though not in other lymphoid cells. Another unusual finding was the apparent presence of hybrid antibody molecules in sera of some mice, with one light chain derived from the rabbit and the other from the mouse. In addition, both μ and γ mouse heavy-chain genes were associated with rabbit light-chain genes.

Insertion of unrearranged chicken λ genes into mice yields similar results, with rearrangement and expression in spleen, and some rearrangement detected in thymus (Bucchini *et al.*, 1987). These data indicate that signals for rearrangement are highly conserved and function even across different classes of organisms. The rearrangement of the foreign gene in thymus suggests the presence of a fastidious discriminatory mechanism in the thymus that prevents endogenous κ-gene rearrangement, a mechanism circumvented by transgene insertion. Finally, the ability of rabbit light-chain genes to associate with mouse μ and γ genes, and the ability to form hybrid molecules with mouse light-chain genes indicate that ontogenic regulation of expression as well as allelic exclusion mechanisms are not functional for unrearranged foreign genes, at least when such genes are derived from heterologous species.

B. Major Histocompatibility Complex (MHC) Genes

The MHC determinants, which, when presented to T cells in the presence of foreign antigen, elicit an immune response, have also been inserted into transgenic mice. Murine and human class I and class II MHC genes (Pinkert *et al.*, 1985; Le Meur *et al.*, 1985; Yammamura *et al.*, 1985;

Bieberich *et al.*, 1986; Krimpenfort *et al.*, 1987) and porcine class I genes (Frels *et al.*, 1985) inserted into mice exhibit appropriate tissue-specific, IFN-inducible regulation, although Pinkert *et al.* (1985) did observe expression of a class II gene in liver, kidney, and heart of one transgenic line.

Two features of the MHC genes that make them of particular interest for insertion into mice are that inbred mice can be used that lack some of the MHC genes. These genes can then be replaced as a model for gene therapy (Le Meur *et al.*, 1985; Yammamura *et al.*, 1985; see also Section VI,C). Another intriguing use of these genes is to direct their expression to inappropriate tissues. Then, when expressed coordinately with tissue-specific cell surface proteins, they may have the potential to present "self" antigens as foreign, and thus to instigate autoimmune rejection of host cells (Sarvetnick *et al.*, 1988; see also Section VI).

The potential for MHC determinants to elicit an autoimmune response underscores the importance of self-tolerance to these proteins. Widera *et al.* (1987) inserted an MHC class II (I–E) antigen gene into mice that carried 1.5 kb of flanking material at the 5′ end and 0.5 kb at the 3′ end. This clone was not expressed in B cells but was expressed in macrophages and dendritic cells. When 2 kb of 5′-flanking DNA was included on the clone, B cells did express, results that localize a B-cell enhancer element between −2 and −1.5 kb (Widera *et al.*, 1987). Of particular relevance to the issue of self-tolerance was the additional finding that when plasmid sequences *(pBR327)* were included on the clone with only 1.5 kb of 5′ DNA, expression was inhibited further and only occurred in thymus. In mixed-lymphocyte cultures, these latter transgenic mice appeared tolerant to I–E antigens, indicating the MHC expression in the thymus is sufficient to induce self-tolerance. Lo *et al.* have reported tolerance to MHC antigens expressed in the pancreas but not the thymus. This finding, if confirmed and extended by further experiments, may revise concepts of MHC-mediated recognition of "self" antigens.

C. Summary

Immunoglobulin heavy-chain genes, light-chain genes, and MHC genes introduced into mice can express at high levels in a tissue-specific manner. Elements regulating tissue-specific light-chain expression may be located as far as 6 kb 3′ to the gene (Storb, 1987), and full expression of MHC genes probably requires as much as 2 kb of 5′-flanking sequence (Widera *et al.*, 1987). Rearranged Ig heavy-chain and light-chain genes in mice can exert allelic exclusion on host loci. In the case of heavy chains, the protein must be membrane bound for allelic exclusion to take place, while allelic exclusion by light chains depends on association of the protein with heavy chains in such a way as to saturate the available heavy-chain protein. Rearranged heavy-chain genes are consistently expressed inappropriately in T cells and are sporadically expressed in other abnormal sites. Rearranged heavy-chain genes profoundly affect immune-system regulation and can lead to elimination of major B-cell subpopulations. Unrearranged Ig genes can be rearranged in transgenics, and such rearrangement can occur inappropriately in T cells. Rearrangement and expression of foreign Ig genes inserted in the germ-line configuration appears not to affect the allelic exclusion mechanism. Ig genes seem not to be inhibited in their expression by cloning vector sequences (Storb, 1987).

MHC gene expression in the thymus appears sufficient to induce self-tolerance. The various sites of MHC gene expression are determined by independent enhancer elements with a B cell-specific element residing 1.5–2 kb 5′ to the gene. MHC transgenics can be used to broaden the immune-response capability of animals and to create mouse models of human disease.

V. Transgenic Mice in the Study of Neoplastic Disease

The ability to insert potentially oncogenic coding sequences into the mouse has allowed a novel approach to investigation of neoplasia. Linkage of such genes to appropriate promoter–enhancer elements can induce high levels of expression in specific tissues, which in turn can cause "directed tumorigenesis." These strategies have provided a wealth of information regarding the oncogenic potential of genes, and the sequence of events that leads normal cells down the path toward malignant transformation. These investigations can be conveniently divided into two categories: insertion of viral oncogenes into mice, and insertion of endogenous protooncogenes and their transformed counterparts into the germ line. Both types of experiments have revealed oncogenic potential of genes as well as established systems for directing tumor formation to specific tissues. Introduction of a variety of other genes into mice has resulted in abnormal cellular proliferation. These experiments will also be considered in this section.

A. Tumorigenesis

1. *Viral Oncogenes*

Transgenic mice provide an excellent test system for viral oncogenesis. Because introduction of viral genes directly into the pronucleus

circumvents barriers to viral infection, pathogens normally unable to infect mouse cells can be studied.

The first viral oncogene to be introduced into mice was the T antigen of SV40. The SV40 T-antigen gene is one of the early-region genes of SV40, and is closely associated with an enhancer element, the 72-bp repeat. In addition, T antigen stimulates activity of the SV40 origin of replication. When this gene with its own promoter and enhancer were introduced into mice, transgenic animals developed choroid plexus tumors (Brinster *et al.*, 1984; Palmiter *et al.*, 1985a; Small *et al.*, 1985). In addition, several mice developed thymic hyperplasia but no malignancies of the thymus. It is of interest that the brain was the site of tumorigenesis, because in early studies involving introduction of the entire SV40 genome into mouse blastocysts, Jaenisch (1974) observed that the greatest amount of SV40 DNA was consistently found in brain. The combined findings suggest that the SV40 early promoter, previously thought to be nonfunctional in mouse cells, can function if the T antigen is present. Expression of T antigen at the RNA level was low in all somatic tissues prior to tumorigenesis, but was high in tumor cell lines (Brinster *et al.*, 1984), suggesting a direct role for the T antigen in oncogenesis. Small *et al.* (1985) made the additional interesting observation that cells explanted to culture from the transgenic animals became progressively more transformed in phenotype and expressed high levels of T antigen. Taken together, these results suggest that a low level of T antigen stimulates proliferation of cells, and, if its expression is high enough or another insult is sustained, causes malignant transformation. An alternative hypothesis is that cells of the choroid plexus are particularly sensitive to the presence of T antigen and are transformed even when expression is low.

The idea that, if high enough, T-antigen expression can lead to transformation of any cell type has been given substantial support in further experiments wherein T antigen has been linked to heterologous promoters. Messing *et al.* (1985) introduced the SV40 T antigen in association with both the 72-bp repeat and the mouse MT-1 promoter. When both regulator elements were intact, the animals developed choroid plexus papillomas as occurred with the 72-bp repeat alone (Brinster *et al.*, 1984; Palmiter *et al.*, 1985a Small *et al.*, 1985). However, when a large portion of the 72-bp repeat was deleted, the MT-1 promoter took control of the T-antigen gene and the animals developed hepatocellular carcinomas (Messing *et al.*, 1985). Thus, when the T antigen is stimulated by a promoter that favors liver expression, the liver is transformed. Hanahan (1985) further demonstrated this point by linking the rat insulin II 5′ region to SV40 T antigen. These transgenics developed cancer of the endocrine pancreas. When targeted to exocrine pancreas by the elastase-I promoter, T antigen induces malignancies of this tissue (Ornitz *et al.*, 1985b, 1987), and when combined with the Ig heavy-chain enhancer and SV40 promoter, the gene induces a wide variety of malignancies (Suda *et al.*, 1987). A particularly persuasive example of the tumorigenic potential of this gene is its ability to induce tumors of the ocular lens, a tissue that does not normally undergo transformation, after linkage to the α-crystallin promoter (Mahon *et al.*, 1987). The proliferative response to T antigen was underscored by experiments with the insulin promoter. Proliferation of pancreatic islet cells was so excessive in these transgenics that the exocrine pancreas was obliterated (Hanahan, 1985). In further studies of these animals, Teitelman *et al.* (1988) observed that islet cells that produce tyrosine hydroxylase as well as insulin escape their normal developmental pathway to senescence and are immortalized, again attesting to the proliferative potential of T antigen-producing cells.

Some evidence also exists that the T antigen exhibits some tissue tropism; that is, the ability of this protein to induce tumors depends on the sensitivity of the target tissue. When linked to the promoter region for atrial natriuretic factor, T antigen is expressed in both the right and left atria of mice. The left atrium is not obviously affected by expression, but the right atrium undergoes pronounced hyperplasia, reaching a several hundred-fold increase in mass before the animals die from cardiac arrhythmias (Field, 1988). In neither atrium do tumors develop, although it is possible that limited survival of the animals does not allow for full expression of the tumorigenic potential of T antigen at this site. When linked to the mouse mammary tumor virus (MMTV) long terminal repeat (LTR), a promoter–enhancer element previously shown to drive expression of the herpes virus *tk* gene in mammary gland and testis (Ross and Solter, 1985), SV40 T antigen was expressed in epithelial cells of lung, kidney, prostate, salivary gland, and mammary gland, and was also expressed in Leydig's cells and lymphoid cells. However, tumors developed only in the ovarian hilus, Leydig's cells, pre-B cells, and seminal vesicle, with dysplasia noted in mammary gland and salivary gland tissues (Choi *et al.*, 1987). This again indicates some tissue tropism for the T antigen.

Other oncogenic viruses also induce pathology in transgenics. BPV induces skin tumors in cattle that are stimulated by surface wounds. Introduction of this virus into mice causes a very similar pattern of pathology to that seen in cattle (Lacey *et al.*, 1986). When inoculated into newborn mice, polyomavirus induces tumors in a wide variety of tissues. Introduction of the large-T antigen into transgenic mice has no effect; however, transgenics carrying the middle-T oncogene develop vascular endothelial tumors (Bautch *et al.*, 1987; Williams *et al.*, 1987). These multifocal vascular tumors often killed mice. Therefore, to improve the opportunity for

studying these neoplasms, Williams *et al.* (1987) transfected the gene into ES cells, selected for expression, and used expressing cells to produce chimeric animals.

A strong tissue tropism was demonstrated for polyoma middle-T antigen in these experiments. The gene was expressed in both vascular endothelium and testis, and was found to be complexed to a tyrosine kinase in both tissues. Yet testes developed no pathology, while multifocal tumors developed in vascular endothelium (Lacey *et al.*, 1986).

Transgenic mice have been used to reveal the oncogenic potential of viral genes. The human papovaviruses JC and BKV are closely related to SV40, with 70–80% homology existing between T-antigen genes from these agents and the SV40 T antigen. JC virus has been associated with progressive multifocal leukoencephalopathy (PML) as well as with glial tumors. BKV has been correlated with subclinical renal infections. When introduced into mice, JC virus induces a dysmyelination syndrome (see Section VI) but also causes adrenal neuroblastomas (Small *et al.*, 1986a). In transgenic mice, BKV induces primary renal, hepatic, and lung carcinomas (Small *et al.*, 1986a).

Another salient example of the use of transgenic mice for demonstrating oncogenic potential of genes comes from experiments with human T-lymphotrophic virus type I (HTLV-I). This virus has been suspected as an inciting agent in human T-cell leukemia. However, fewer than 0.1% of individuals with antibodies to HTLV-I develop leukemia, and the latent period for development of malignant disease can exceed 20 years. When T-cell leukemia develops, no viral antigens can be detected on leukemic cells.

One HTLV-I gene, the *tat* gene, encodes a protein that induces the viral LTR to stimulate viral gene expression. This gene was linked directly to the viral LTR and inserted into transgenic mice (Hinrichs *et al.*, 1987; Nerenberg *et al.*, 1987). Transgenic mice did not develop leukemia but did develop mesenchymal tumors shown to be neurofibromas (Hinrichs *et al.*, 1987). Thus, these mice demonstrated the oncogenic potential of HTLV-I and further showed that the *tat* gene is by itself sufficient to induce tumors.

2. *Immunobiology of Foreign Oncogene Expression*

Expression of foreign oncogenic proteins in transgenic mice raises questions concerning the issue of immune surveillance. Why are tissues that express such genes not rejected by virtue of the presence of the foreign protein? Faas *et al.* (1987) found that mice that develop tumors and carry SV40 T antigen are tolerant to the T antigen, and Adams *et al.* (1987) showed that in mice expressing T antigen in the pancreatic islets, variable degrees of islet cell rejection occur. The results suggest that early expression of T antigen is associated with immune tolerance and allows the tumorigenic potential of the protein to be realized, while later production of the antigen elicits an immune response that can kill cells before they become transformed.

3. *Cellular Protooncogenes and Their Oncogene Counterparts*

The protooncogene c-*myc* has been introduced into transgenics to investigate directly its tumorigenic potential. When inserted with the MMTV promoter–enhancer, a steroid-responsive element known to be active in breast tissue, multiparous mice developed breast tumors (Stewart *et al.*, 1984). Not all mammary cells became malignant, and expression of the transgene in salivary gland and intestine was not associated with pathology. In one of these lines, the MMTV–*myc* construct was expressed in a wider variety of tissues. In this line, tumors of breast, testis, and B and T lymphocytes were seen (Leder *et al.*, 1986). This pattern of tumor development corresponds well with the distribution of a reporter gene linked to the MMTV LTR (Ross and Solter, 1985). When c-*myc* was linked to Ig enhancer elements, lymphoid malignancies developed (Adams *et al.*, 1985; Suda *et al.*, 1987). These data indicate that if inappropriately active, the c-*myc* protooncogene can predispose to development of malignant disease.

Similar experiments have been performed with *ras* oncogenes and protooncogenes. Mutated *ras* oncogenes linked to the SV40 promoter and Ig heavy-chain enhancer cause adenomatous lung tumors (Suda *et al.*, 1987), and, when linked to the whey acidic protein promoter, breast tumors (Andres *et al.*, 1987). Quaife *et al.* (1987) linked the c-H-*ras* oncogene to the elastase-I promoter and produced transgenic mice. On days 16–20 of gestation, these animals developed massive tumors of the acinar cells, which arose within a few days of acinar cell differentiation. These tumors were associated with severe disorganization of the organ structure and formation of cysts; however, cells of the endocrine pancreas could still be distinguished and were found to be disorganized in arrangement but not part of the tumor. In addition to tumor formation, acinar cells appeared thwarted in their differentiation. They never developed eosinophilic granules characteristic of the mature acinar cell, and appeared arrested at a stage of differentiation normally characteristic of day 14 fetal exocrine pancreas.

When the normal *ras* protooncogene linked to elastase-I was inserted as a control (Quaife *et al.*, 1987), subtle anomalies were present, including appearance of multifocal areas of large eosinophilic cells, hyperplastic acini, and some anaplastic cells with multiple nuclei. These data indicate that overexpression of *ras* disturbs the balance between cell division and

differentiation, inhibiting differentiation. Also of interest in this report was the finding that *myc* linked to elastase-I was expressed in the exocrine pancreas at a similar level to *ras*, but no pathology developed. This could indicate some negative tropism with regard to the oncogenic potential of *myc* in acinar cells, or that, even with the elastase-I promoter, expression was not high enough to induce pathology.

Sinn *et al.* (1987) have introduced the v-Ha-*ras onc* gene into mice driven by the MMTV LTR. These animals developed benign diffuse hypertrophy of the harderian lacrimal gland, and also focal mammary, salivary, and lymphoid tumors. Because the entire harderian gland was hyperplastic, while only focal areas of the other affected tissues manifested pathology, the authors concluded that *ras* was by itself sufficient to induce harderian gland hypertrophy, but that the focal malignancies seen at other sites required one or more additional somatic events.

This report also investigated the interaction of MMTV/v-H-*ras* and MMTV/c-*myc* transgenes in one animal by crossing independently generated lineages to produce double carriers of the transgenes. These animals exhibited a dramatic acceleration of tumor formation relative to either single carrier strain alone, suggesting a synergistic action of the two transgenes which favored tumorigenesis (Sinn *et al.*, 1987).

4. *Mechanisms of Tumor Formation in Transgenic Mice Carrying Oncogenes*

These transgenic mice have been used to attempt to address a fundamental question regarding the transition of cells from normal to malignant: is a single insult, or "hit," sufficient to induce malignancy, or are at least two events required? The pattern of pathology in mice with SV40 T-antigen genes and with *myc* genes suggest at least a "two-hit" mechanism (Hanahan, 1985; Sinn *et al.*, 1987). In these animals, tumors arise rarely among either a group of hyperplastic cells or normal cells. Because the transgenes are active in all cells of the target organ, it must be inferred that the introduced gene provides the first insult, which renders many cells abnormal, and a few cells sustain a second event, which precipitates the transition to malignancy.

The situation with *ras* and polyoma genes is not as clear. In mice with elastase-I-*ras* genes, the entire exocrine pancreas was affected, and in chimeric mice carrying cells with the polyoma middle-T antigen, multifocal vascular endothelial tumors appeared, again suggesting that all cells degenerated into tumors (Williams *et al.*, 1987). These patterns are not inconsistent with a model in which transgene expression is alone sufficient to convert entirely normal cells into cancer cells. However, data from *ras* transgenics is inconsistent, with clear evidence in some animals of a multiple-hit mechanism (Sinn *et al.*, 1987). At present I do not believe that a one-hit model of tumorigenesis has ever been unequivocally demonstrated in transgenic animals. Multifocal tumor formation as seen with the polyoma middle-T antigen could be attributed to a relatively high sensitivity of cells to a second insult, or could indicate that any one of a variety of second events is sufficient to cause neoplasia in these cases.

B. Other Proliferative Disorders

As noted previously, introduction of the SV40 T-antigen gene linked to atrial natriuretic factor does not cause maligancy, but massive hypertrophy of the right atrium (Field, 1988). A similar effect has been seen with SV40 T antigen linked to the GH-releasing factor promoter. Here, massive thymic hyperplasia was seen (Botteri *et al.*, 1987). Overexpression of the c-*fos* protooncogene causes abnormal enlargement of the metaphysis of bones (Ruther *et al.*, 1987). Finally, in several transgenic mice with tumors, hyperplasia without neoplasia is seen in various nonmalignant tissues (Brinster *et al.*, 1984; Sinn *et al.*, 1987).

Abnormal cell growth has been noted not only in response to oncogene expression, but also from abnormally elevated or inappropriately targeted expression of normal genes. Chen *et al.* (1987) introduced the Thy-1 gene into mice linked to the Ig heavy-chain enhancer. Thy-1 is a cell surface protein of the Ig supergene family. In mice this gene is expressed in thymocytes, brain, and peripheral T cells, but not in bone marrow (see, e.g., J. W. Gordon *et al.*, 1987). When Thy-1 is driven to express in bone marrow by the heavy-chain enhancer, hyperplasia of bone marrow and lymph nodes is seen in association with Thy-1 expression in the B-cell lineage. Chen *et al.* (1987) proposed that Thy-1 is a differentiation antigen which, because of its inappropriate expression, induces abnormal proliferation within the B-cell lineage.

Kollias *et al.* (1987) obtained similar results with human Thy-1 linked to mouse 5′ and 3′ regions. These animals expressed Thy-1 abnormally in kidney and consequently developed a proliferative disorder of tubular epithelium. In addition, one animal had a brain tumor, which is particularly surprising, since Thy-1 is normally expressed throughout the brain of both mouse and human. We have introduced the intact human and mouse Thy-1 genes into mice as well (J. W. Gordon *et al.*, 1987), and have noted no pathology, this despite expression of human Thy-1 in a variety of tissues that are abnormal sites for Thy-1 expression in mice. The reason for this disparity is not yet clear.

A lymphoproliferative abnormality has been induced in transgenics by linkage of the gene for the hematopoietic growth factor GM-CSF to a

retroviral promoter (Lang *et al.*, 1987). These animals expressed the transgene in the eye, peritoneal cells, and striated muscle, which became infiltrated with activated macrophages. As a result of expression, macrophages accumulated in muscle, peritoneum, and eye, and the animals developed retinal damage and fatal muscle wasting. *In vitro* studies revealed no increase in the macrophage pool or in proliferative capacity. Therefore, these investigators proposed that elevated GM-CSF increases macrophage survival and results in fatal accumulation of these cells (Lang *et al.*, 1987).

C. Summary

Inappropriately elevated or targeted expression of oncogenes or protooncogenes induces a variety of heritable neoplastic disorders in transgenic mice. In addition, other mammalian genes can alter control of cell populations by increasing proliferative capacity or cellular half-life. These experiments show that inappropriate regulation of normal cellular genes can induce maligancy, and that abnormal expression of foreign or endogenous genes can disrupt normal mechanisms that regulate cell number and balance cell death with renewal. The ability to induce such disorders in a developmentally timed and tissue-specific manner has contributed significantly to understanding of neoplastic disease.

VI. Transgenic Mouse Models of Human Disease

Introduction of genes into mice can induce disease states by either of two mechanisms: foreign gene expression or insertional mutagenesis. Expression can cause disease when genes from viral pathogens are expressed in mice, when expression of endogenous genes is inappropriately targeted, when gene dosage is disturbed by addition of a new coding sequence, or when mutant genes with dominant effects are inserted. Disease models are created by insertional mutagenesis when introduction of new DNA disrupts a mouse gene, mutation of the human counterpart of which is associated with human disease. A variety of abnormalities have been observed in transgenic mice that do not directly represent human disease states. These anomalies will be dealt with elsewhere. In this section only those abnormalities that closely represent previously characterized human disease states will be discussed. Abnormalities relating to gene expression will first be reviewed, followed by discussion of potential models created by insertional mutagenesis. In some cases biochemical defects have been created that precisely represent similar disorders in human diseases. Even

though these animals do not always manifest pathology, the ability to create such defects allows investigation of many aspects of the human disease.

A. Gene Expression

1. *Down's Syndrome*

Down's syndrome, or trisomy 21, is a common chromosomal abnormality in humans and is associated with a variety of developmental disorders including mental retardation, cardiac defects, predisposition to malignancy, and accumulation of neuritic plaques in the brain. These problems are all presumed to result from elevated dosage of genes linked to chromosome 21, but because of the large number of genes involved in the trisomic state, it has not been possible to determine which or how many of the genes must be represented in triplicate for the stigmata of Down's syndrome to appear.

As an approach to this question, Epstein *et al.* (1987) inserted the gene for Cu/Zn superoxide dismutase (SOD) into mice. This gene maps to chromosome 21 and is often triploid in Down's syndrome. When the human gene was inserted into mice, it expressed in a manner similar to that of humans, with 0.9- and 0.7-kb transcripts present in a 1 : 4 ratio. Human enzyme was synthesized, and its peptides formed heterodimers with mouse SOD. Total SOD activity was increased 1.6- to 6-fold in brains of all four lines tested, indicating that no feedback regulation occurred. Expression was also detected in heart, lung, and liver, and in red blood cells of two lines. These transgenics manifested no pathology (Epstein *et al.*, 1987). However, the fact that transgene insertion elevated the total enzyme activity in the animals indicates that transgenic technology may be used in further experiments to identify those genes whose abnormally elevated expression leads to deformities typical of trisomy 21. This observation is important because it is not true for all transgenes. Transgenic mice carrying insulin genes, for example, exhibit normal glucose homeostasis (Selden *et al.*, 1986).

2. *The Hepatitis B Surface-Antigen Carrier State*

Hepatitis B is a common and serious viral disease. After hepatitis B infection several outcomes are possible, including fulminant hepatic failure and rapid death, complete recovery, chronic active hepatitis with slow destruction of the liver, and persistence of viral antigens in blood in the absence of disease. The latter surface-antigen carrier state has been mimicked in mice by introduction of the hepatitis B surface antigen into mice (Chisari *et al.*, 1985; Babinet *et al.*, 1985). These animals synthesized surface-antigen protein in liver as well as other organs, and surface antigen

could be detected in blood. In some animals, antibodies were also present. These animals thus may constitute a mouse model for study of the surface-antigen carrier state.

3. *Progressive Multifocal Leukoencephalopathy (PML)*

Progressive multifocal leukoencephalopathy is a neurodegenerative disorder that has been associated with JC virus infection. Introduction of JC virus into transgenic mice (Small *et al.*, 1986b) resulted in T-antigen expression in the central nervous system with an associated dysmyelination syndrome bearing similarities to PML. These animals may thus provide a model for study of PML, and also establish a clear relationship between JC virus infection and dysmyelination.

4. *Neurofibromatosis*

Neurofibromatosis is an inherited disorder characterized by tumors of the nerve sheaths. When Nerenberg *et al.* (1987) introduced the *tat* gene of HTLV-1 into transgenics, animals developed multiple mesenchymal tumors. Hinrichs *et al.* (1987) identified these tumors to be neurofibromas, and thus propose that these transgenics constitute a model for neurofibromatosis.

5. *Osteogenesis Imperfecta*

Osteogenesis imperfecta is a fatal, dominantly inherited disorder of collagen formation. The normal collagen is a triple-helical protein composed of two α_1 chains and one α_2 chain. The α chains are simple proteins, with a glycine residue appearing at every third position. In osteogenesis imperfecta, an arginine substituted for a glycine at position 391, or a cysteine substituted for a glycine at positions 748 or 988 of the α_1 chain interferes with formation of the triple helix. The result is markedly reduced formation of mature collagen molecules and death. Stacey *et al.* (1988) employed site-directed mutagenesis to substitute a cysteine or arginine at position 849 of the α_1 gene and microinjected the mutated construct into mice. Seven of nine animals that died neonatally were transgenic. When transgenic fetuses were examined, 10% of the mutant gene RNA was sufficient to reduce type I collagen to 46% of normal. These animals thus constitute a model for osteogenesis imperfecta, and reproduce the dominant mutant phenotype.

6. *Insulin-Dependent Diabetes Mellitus*

Some cases of insulin-dependent diabetes mellitus are associated with pancreatic islet cell destruction. This pathology has been thought to have an autoimmune basis. Inappropriate expression of class II MHC determinants on islet cells could result in false presentation of islet cell surface antigens as foreign, and lead to destruction of the islet cell. Such inappropriate expression of MHC could secondarily result from infection, which might be associated with locally elevated levels of IFN, a substance known to induce MHC gene expression. Insulin-dependent diabetes can be a sequela of infection-related pancreatitis.

To test these suppositions, Sarvetnick *et al.* (1988) introduced two kinds of constructs into transgenics. First, to induce MHC expression in islet cells directly, these genes were linked to the insulin promoter. Second, IFN genes were linked to insulin regulators in an attempt to induce MHC expression secondarily. In both transgenics, insulin-dependent diabetes developed in association with loss of pancreatic islets. In the MHC mice, islet atrophy was pronounced, while in the IFN mice, an inflammatory response leading to islet destruction was apparent (Sarvetnick *et al.*, 1988). These data clearly establish that inappropriate expression of MHC or IFN genes can lead to diabetes mellitus, and the animals may serve as a useful model for this human condition. Lo *et al.* (1988) obtained similar results with MHC genes, but further found that transgenic mouse T cells were tolerant to the foreign antigen, and, in agreement with Sarvetnick *et al.* (1988), found no evidence of lymphocytic infiltration. They accordingly suggest that inappropriate MHC expression is not by itself sufficient to cause autoimmune diabetes mellitus.

7. *Ankylosing Spondylitis*

Ankylosing spondylitis is an autoimmune arthritis common in individuals with the HLA B27 HLA type. Mice expressing HLA B27 were produced by introducing the B27 heavy chain into one line, β_2-microglobulin into another, and crossing the animals to produce double carriers. The genes were appropriately expressed in a manner biochemically indistinguishable from human cells, thus establishing a potential animal model for this disease (Krimpenfort *et al.*, 1987).

B. Disease Models Relating to Insertional Mutagenesis

Lesch–Nyhan syndrome is an X-linked disorder characterized by mental retardation, spastic cerebral palsy, choreoathetosis, and bizarre behavior patterns including self-mutilation. The disease results from a deficiency of HPRT, although the mechanism by which the enzyme deficiency leads to these symptoms is unknown, and no animal model for HPRT deficiency exists. Using the ES-cell gene transfer system, Hooper *et al.* (1987) and Kuehn *et al.* (1987) produced animals deficient in HPRT. Hooper *et al.* grew ES cells in the presence of 6-TG, which, by virtue of HPRT action,

can be substituted for purines in DNA and kill the cell. Surviving cells ($HPRT^-$) were then used to make chimeras and subsequently contributed to the germ line to produce animals totally deficient in HPRT. Using a similar approach, Kuehn *et al.* (1987) first infected ES cells with retrovirus, then selected with 6-TG. Although the animals were not abnormal, they demonstrate the utility of the ES system for creating mutations identical to those found in human diseases.

The association of foreign viral gene expression with disease in humans can be duplicated in transgenic mice. In addition, expression of mutant genes that manifest as dominant mutations can be reproduced in these animals. Insertion of exogenous DNA apparently can put any endogenous gene at risk for mutation, and thus, insertional mutagenesis has great potential for duplicating many recessive mutations. The ES-cell system may also be used in the future to create specific recessive mutations. Although homologous recombination in mammalian cultured cells is a rare event, the ability to transfect large numbers of ES cells *in vitro* raises the prospect of mutating specific genes by homologous recombination, then reinserting the mutant cells into mice by producing chimeras. This technology would improve enormously the study of genetic disease, and awaits only an efficient method for detecting homologous recombination in cultured cells.

C. Transgenic Mice as Models for Gene Therapy

In addition to their use for creation of disease models, transgenic mice can be used as models for gene therapy of disease. In these cases, the treated conditions do not always have precise counterparts in humans. However, the ability to correct deficiencies by gene insertion is still instructive with regard to the potential for gene therapy, because it allows study of both the power and limitations of the gene therapy approach. Thus, information developed from these animals has impact on future consideration of germ-line gene therapy.

1. *Gene Therapy in the Immune System*

Le Meur *et al.* (1985) injected Ia-antigen genes into mouse embryos. These MHC class I genes are inducible by IFNs and are involved in presentation of foreign antigens to T cells. *Ia* genes fall into two complexes (A and E), and C57BL/6 mice lack an intact *Ia* gene of the E complex. This deficiency leads to the inability to respond to the synthetic antigen poly Glu-Lys-Phe (GLϕ).

When an intact E complex *Ia* gene was inserted, expression was inducible and tissue-specific, and the animals were responsive to priming with GLϕ. These data indicate that insertion of *Ia* genes can confer immune responsiveness to organisms with deficiencies of *Ia* genes. Similar results were simultaneously reported by Yammamura *et al.* (1985).

2. *Gene Therapy in the Endocrine System*

The ability to induce increased somatic growth of mice by insertion of MT–GH constructs (Palmiter *et al.*, 1982a, 1983a) led Hammer *et al.* (1984) to attempt to correct GH deficiency in mice by gene transfer. MT–GH genes were inserted into *lit/lit* mice, which are growth retarded and male infertile due to lack of GH production. The transgenic mice grew even faster than normal, thus correcting the growth retardation. However, although male fertility was restored, female transgenics were infertile, probably because of the tonic pattern of GH production from the transgene (Hammer *et al.*, 1984).

Mason *et al.* (1986) have microinjected the gonadotrophin-releasing hormone (GnRH) gene into hypogonadal (*hpg*) mice, which are sterile due to a deficiency in GnRH. When this gene was inserted into *hpg* carriers and then crossed onto the homozygous background, *hpg/hpg* transgenics produced normal pituitary and serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin. Immunocytochemistry and *in situ* hybridization showed GnRH expression in the appropriate hypothalamic neurons, with a small amount of ectopic expression. Moreover, these animals were able to reproduce, and their genetic deficiency was thus corrected.

3. *Gene Therapy in the Hematopoietic System*

Costantini *et al.* (1986) inserted the mouse β-major globin gene or the human β-globin gene into mice afflicted with β thalassemia as a result of a deletion of the β-globin gene. Introduction of the transgenes was associated with tissue-specific expression. Some animals showed complete correction of all abnormalities associated with the thalassemic condition. Moreover, human β-globin was able to rescue the mouse defect by combining with mouse α chains to produce a functional globin molecule.

4. *Gene Therapy in the Nervous System*

Mice homozygous for the spontaneous mutation *shiverer* are unable to produce myelin basic protein (mbp) because of a partial gene deletion. These mice normally die at an early age. Insertion of an intact cosmid clone of the *mbp* gene with 4 kb of 5′- flanking material resulted in normal temporal, spatial, and developmental regulation of the gene, with expression in neural tissue beginning at 1 month of postnatal life (Readhead *et al.*, 1987). In addition, normal differential splicing of the *mbp* gene was reproduced for the transgene. Transgenics expressed up to 25% of the normal amount of *mbp* with the degree of correction of the shiverer phenotype correlating well with the level of expression (Popko *et al.*, 1987). The transgenic animals were restored to a normal life span.

5. *Genetic Manipulation of Lipid Storage and Transport*

While not strictly an example of gene therapy, mice produced by Hoffmann *et al.* (1988) suggest potential for gene transfer as a method of treating disorders of lipid storage and transport. These investigators produced transgenic mice carrying a MT–LDL receptor gene, and induced gene expression with cadmium injections. As a result the mice produced excess LDL receptor, and the amount of free LDL is in the bloodstream plummeted. Because a relative excess of LDL in blood correlates with coronary vascular disease, these animals raise the intriguing prospect that gene transfer might be used to alter the amounts of such substances and reduce risk of disease.

D. Summary

Tissue-specific and developmentally regulated expression typically characteristic of foreign genes in transgenic mice makes gene transfer for correction of genetic deficiencies an attractive prospect, and several successful experiments have been performed. The importance of the level of expression to correction of the mutant phenotype, and the consequences of ectopic expression remained to be determined in each case. In addition, before such procedures can ever be used in medicine, gene transfer frequency must be increased, and the disruptive effects of foreign gene insertion must be eliminated (see Section X).

VII. Special Applications of Transgenic Mice

In addition to the aforementioned broad applications of transgenic technology, occasional findings have been reported that have fundamental implications for our understanding of developmental genetics, or that suggest that an important new major use of such animals is emerging. This somewhat eclectic but significant group of findings is summarized here.

A. Genetic Studies

1. *Transgenic Mice Reveal the Importance of Parental Gene Imprinting*

In adults, both alleles of every active gene appear to be equally functional. What difference would it then make if all genes are inherited from a single parent? Markert and Petters (1977) explored this question by removing one of the two pronuclei from fertilized mouse eggs, then diploidizing the haploid zygote by inhibiting the first cleavage division with cytochalasin B. These embryos cleaved to the blastocyst stage, although no live young were reported born after embryo transfer. Hoppe and Illmensee (1977) later reported birth of mice from this experiment and thus asserted that homozygous uniparental animals, either gynogenetic (derived from the female only) or androgenetic (derived from the male), were viable. Later, however, simpler techniques for generating androgenetic and gynogenetic embryos in large numbers were devised, and in every case, uniparental embryos, even when not homozygous, were developmentally inviable (Surani and Barton, 1983; McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Surani *et al.*, 1984; Barton *et. al.*, 1984). Stevens *et al.* (1977) and Anderegg and Markert (1986) did find that uniparental embryo cells could be rescued in chimeric mice. Anderegg and Markert (1986) proposed from these and other data that differential methylation of maternal and paternal genes might be responsible for differences in activity of a subset of genes that in turn are important for normal development.

Transgenic mice have provided strong support for this hypothesis. Swain *et al.* (1987), in studies of mice carrying the c-*myc* gene linked to the MMTV LTR, found one line that expressed the transgene in heart, but only if that transgene was inherited from the male parent. If inherited from the female the gene was not expressed. This difference in expression correlated with heavy methylation of the gene when inherited from the female and corresponding undermethylation of the paternally inherited transgene. Similar results were reported by Hadchouel *et al.* (1987) for a line of transgenic mice carrying the HBsAg gene. In one of their lines only transgenic males sired progeny that expressed the transgene, and when the foreign gene was transmitted through females its expression was permanently extinguished. This repression was correlated with markedly increased methylation. These data thus provide compelling support for the notion that differential methylation of paternal and maternal genes may result in the requirement that embryos receive genetic information from both sexes. Independent of the issue of gene expression, Reik *et al.* (1987) found that one of seven transgenic lines carrying the bacterial CAT gene showed increased methylation after transmission through the female germ line, and similar results were reported for four of five lines carrying a quail troponin gene (Sapienza *et al.*, 1987). In all cases, it is female germ-line transmission that increases methylation.

It is not presently known which loci normally undergo differential methylation. Obviously this mechanism is not invoked for all genes or even all transgenes. It remains an important goal to identify, clone, and characterize these differentially expressed genes and determine their roles in normal development.

2. *Transgenic Mice and X-Chromosome Inactivation*

X-chromosome inactivation, or Lyonization, is the mechanism by which female (XX) mammalian cells reduce the activity of their X-linked genes to equal that of males (XY). The mechanism of X-chromosome inactivation is not clear. Production of transgenic mice has occasionally resulted in fortuitous integration of foreign DNA into the X chromosome. Studies of expression of X-linked transgenes has provided insights into the X-inactivation mechanism.

Goldman *et al.* (1987) studied an exogenous transferrin gene integrated into the X. Males carrying the transgene were mated with females heterozygous for Searle's translocation, a translocation of part of the X that prevents inactivation of the involved chromosome. Female offspring carrying Searle's translocation were evaluated for transgene activity, because in such animals the X carrying the transgene would be preferentially inactivated. The transgene was active, indicating that transgenes escape X inactivation. Because some regions of the X normally remain active, and these regions manifest frequent crossing over with the Y chromosome, these transgenic mice were evaluated for X–Y exchanges involving the transgene, and none were found. Thus it was concluded that the transferrin gene failed to undergo inactivation despite being integrated into a region of the X that normally undergoes inactivation.

Goldman *et al.* (1987) proposed an explanation for failure of transgene inactivation based on the structure of DNA in the region of the X, which is normally silenced. In this chromatin, GC-rich regions thought to be involved in chromosome inactivation are spaced approximately 100 kb apart. The transferrin transgene was 17 kb in length, and was integrated as a concatameric structure containing more than 10 subunits. Thus, this integrated element, greater than 100 kb, would, by separating the GC-rich domains, be expected to interfere with the inactivation mechanism.

Krumlauf *et al.* (1985a,c) identified a transgenic line in which an integrated α-fetoprotein gene was X-linked and was not inactivated when located on the inactivated X in yolk sac, but was inactivated in fetal liver. This was determined by breeding the X-linked transgene from males and examining expression in yolk sac of fetuses, a tissue in which the paternal X is preferentially inactivated. Even though two other paternally derived alleles, Phosphoglycerate kinase (Pgk^b) and $Hprt^b$, were silent in yolk sac, the transgene remained active. However, when transgenic males were crossed to females carrying Searle's translocation, and liver of transgenic newborns were evaluated, the X-linked transgene was inactivated. No explanation exists at present for this differential inactivation, although several hypotheses including differential methylation were proposed (Krumlauf *et al.*, 1985a). In this instance, the integrated concatamer was about 85 kb (12 copies of a 7-kb insert). It is possible that the insert approximated a threshold size, above which inactivation is blocked. As a result, the gene was only inactivated in a subset of tissues.

On a related subject, Harbers *et al.* (1986) studied a mouse with a retroviral insertion on the pseudoautosomal region of the X, which pairs with the Y and is involved in reciprocal exchanges during male meiosis. Using this insert as a probe to evaluate the DNA in this region, they found that flanking DNA was tandemly repeated and highly variable. In addition, proviral copies were lost or gained in 7% of male meioses, indicating crossing over in the region. The data are consistent with the proposal that unequal recombination occurs frequently in the pseudoautosomal region (Harbers *et al.*, 1986).

3. *Cell Lineage Ablation*

Cloning of genes encoding various toxic products creates the opportunity to direct expression of such genes to specific tissues in transgenic mice and thereby eliminate specific cell lineages. Palmiter *et al.* (1987) linked the gene encoding the diphtheria toxin A polypeptide to the rat elastase-I promoter and inserted the construct into transgenic mice. Transgenics showed variable degrees of ablation of the exocrine pancreas, thus demonstrating the feasibility of the ablation strategy. Breitman *et al.* (1987) used the same approach but employed the γ-crystallin promoter. Again a variable effect was seen, with some transgenic animals showing reduction in lens size and others a total absence of normal lens tissue.

This strategy presents intriguing prospects for destroying specific cell lineages in the developing embryo, or for eliminating neoplastic cells from animals, using the toxin gene as a "genetic magic bullet." First, the appropriate regulatory elements must be identified, and must direct highly tissue-specific expression, as is the case for the elastase and crystallin promoters. Even low-level expression of toxin genes in ectopic sites could interfere with interpretation of results or kill the animal. However, such protocols have significant potential application to studies of development and disease.

B. Genetic Engineering

1. *Genetic Engineering of Chemotherapy Resistance*

One goal of somatic gene therapy is the introduction of chemotherapy resistance genes into bone marrow or other somatic cells in order to render specific tissues resistant to chemotherapeutic drugs. Retrovirus-mediated gene transfer is likely to be used for such efforts, but at present expression of retroviral vectors is not sufficiently high to test whether gene therapy

will lead to an altered physiological response to drug treatment. To make the latter determination, Isola and Gordon (1986) inserted a methotrexate (MTX)-resistant dihydrofolate reductase gene into transgenic mice. Transgenic animals survived significantly longer than controls after MTX treatment, thus indicating that if efficiently expressed, drug resistance genes can increase tolerance to toxic agents.

2. *Genetic Engineering of Agricultural Animals*

For a variety of reasons, production of transgenic farm animals is of potential value (Gordon, 1986b). Hammer *et al.* (1985b) demonstrated the technical feasibility of gene insertion into rabbits, sheep, and pigs by transfering GH genes. While it is not yet clear that such experiments will lead to more rapid growth of economically important livestock, large transgenic animals can serve other purposes. The promoter of whey acidic protein (WAP) can direct expression of heterologous genes to breast tissue (Andres *et al.*, 1987), and has been used by K. Gordon *et al.* (1987) to produce transgenic mice that secrete tissue plasminogen activator in milk. Similarly, sheep β-lactoglobulin is expressed in breast tissue and alters the quality of milk produced by mice (Simons *et al.*, 1987). These and other experiments indicate that large animals can be used as "factories" for production of complex proteins that, because of extensive posttranslational modification, are difficult to synthesize in bacteria. These proteins would be free of a variety of human diseases that could be acquired by harvesting such compounds from human serum.

3. *Tracing Cell Lineages Using Exogenous DNA as a Marker*

Soriano and Jaenisch (1986) infected preimplantation mouse embryos with retroviruses, reimplanted the embryos, and studied fetuses that were mosaics for retroviral integration. Because each integration event can be identified by a unique pattern after Southern hybridization, it was possible to trace the mitotic descendants of a single infected blastomere. By studying the intensity of hybridization in respective tissues, and the percentage of animals with both germ-line and somatic integration, they were able to determine that cells of the embryo proper intermingle extensively, that at most eight cells give rise to somatic lineages, and that prior to somatic allocation at least three cells are allocated to the germ line (Soriano and Jaenisch, 1986). This report demonstrates that foreign DNA can be used in much the same way as genetic markers in chimeric mice were used in the past to trace cell lineages (Mintz, 1974), but that a greater degree of accuracy can be achieved with a molecular approach.

C. Summary

Fortuitous integration of transgenes on the X chromosome has allowed for new and insightful investigations of gene regulation on this chromosome. In addition, use of promoter–enhancer elements to target gene expression to specific tissues has been applied to several genetic engineering experiments. These efforts, in conjunction with gene insertion into large animals, has potential to improve agricultural technology as well as to develop animal models for gene therapy of human disease.

VIII. Insertional Mutagenesis

Insertional mutagenesis occurs when foreign DNA integrates within or close to an endogenous gene with a resultant loss of host gene function. Insertional mutations can occur by retroviral DNA insertions as well as by microinjection, although it is presently unclear whether integration is entirely random and thus, whether all genes are at equal risk for disruptive integration by retroviral and microinjected DNA. The number of insertional mutations reported thus far has been relatively few, and thus it is difficult to ascertain the actual frequency of such events. Estimates range from 7% (Palmiter and Brinster, 1986) to 20% (Scangos and Bieberich, 1987). Factors contributing to underestimation of the frequency include the fact that dominant lethal insertions are not likely to be detected, and that many laboratories do not carefully screen all transgenic lines for insertional mutations. On the other hand, the frequency of abnormalities present after integration of microinjected DNA may exceed that caused by insertional mutagenesis, because integration of microinjected material may be associated with chromosomal translocations and other rearrangements that cause developmental defects but are not, in the strictest sense, insertional mutation events.

A number of methods can be used to distinguish insertional events from defects related to foreign gene expression. The most obvious strategy is to breed the animals and show a pattern of inheritance that both links the transgene to the abnormal phenotype and is typical of a dominant or recessive Mendelian trait. Southern analysis can also reinforce the data: when DNA flanking the insertion site is cloned it can be used as a hybridization probe against DNA from the mutant and normal animals. If foreign DNA insertion creates a restriction fragment-length polymorphism (RFLP), the presence of this polymorphism can also be correlated with the mutant phenotype. In cases of recessive mutation, the number of "doses" of the transgene can be compared in mutant and carrier animals.

and can demonstrate that all mutant animals have twice as much foreign DNA per host genome as carriers. Finally, if a spontaneous mutation exists that can be demonstrated to be allelic with the transgenic insertional mutation, compelling evidence is provided that it is insertion, not expression, that causes the abnormality.

The best-characterized insertional mutation to date resulted from insertion of the Moloney murine leukemia virus (MoMLV). Jaenisch and colleagues originally generated a number of such lines and in one, Mov-13, inbreeding of positive animals was associated with a reduced litter size. Close evaluation later revealed that mice demonstrated by RFLP analysis to be homozygous for the proviral DNA insert died at midgestation, that death was due to interruption of the α_1(I)-collagen gene by insertion of the foreign DNA into the first intron of that gene, and that the insertion event blocked initiation of transcription, probably because it altered that pattern of DNase I-hypersensitive sites at the 5′ end of the gene (Jaenisch *et al.*, 1983; Schnieke *et al.*, 1983; Breindl *et al.*, 1984; Harbers *et al.*, 1984; Hartung *et al.*, 1986). This is the only case in which the gene mutated by insertion has been identified. Another mouse from the same series, Mov-34, causes early postimplantation lethality when homozygous (Soriano *et al.*, 1987). The proviral DNA mapped to the 5′ side of a highly active and ubiquitously expressed gene, although the product specified by the gene has not yet been determined.

Woychik *et al.* (1985) identified a recessive insertional mutation by inbreeding mice carrying the c-*myc* protooncogene linked to the MMTV promoter. In 25% of offspring from heterozygous crosses, animals showed a deformity characterized by fusion of the bones of the limbs. That the mice were homozygous for the foreign DNA insert was demonstrated by frequency of inheritance of the disorder in crosses of affected and carrier animals, and by Southern blot analysis, which identified a RFLP created by transgene insertion. In addition, this mutation was shown to be allelic with a previously identified spontaneous mutation limb deformity (*ld*), which in the homozygous state causes a similar phenotypic defect.

Palmiter *et al.* (1983b) identified an insertional mutation in a line of mice carrying the herpesvirus *tk* gene linked to the MT-1 promoter. In this case, male carriers of the transgene were fertile but unable to transmit the new gene. Southern analysis suggested a paucity of mature sperm carrying the transgene, suggesting degeneration of sperm that inherited the *tk* gene through meiotic segregation of the chromosomes.

Overbeek *et al.* (1986) noted another limb deformity in mice carrying the Rous sarcoma virus LTR linked to the bacterial CAT gene. In this case, 3 of 15 offspring from heterozygous crosses had fused digits, and 100% of progeny from affected animals showed the same defect.

Several other prenatal lethal insertional mutations have been reported (Shani, 1986; Mark *et al.*, 1985; Wagner *et al.*, 1983; Gerlinger *et al.*, 1986). That the defects arose from DNA insertion was documented by a variety of the aforementioned methods. To date, none of the genes involved in these mutations has been identified.

The relative frequency of insertional mutations, and the preponderance of mutations causing prenatal lethality have led to speculation that foreign DNA integrates preferentially into active genes (Wagner *et al.*, 1983; Schnieke *et al.*, 1983). This notion is supported in part by the finding that chromosomal DNA surrounding retroviral insertions often contains DNase I-hypersensitive sites (Vijaya *et al.*, 1986; Rohdewohld *et al.*, 1987). However, the frequency of host gene disruption is not far from what would be expected when one considers that many genes are very large, and that the sequences composing their associated regulatory elements add still further to the percentage of DNA vulnerable to insertional mutagenesis. In addition, as the description of some of the characterized mutants should illustrate, many affected genes are not likely to be open to transcription at the time of exogenous DNA integration. Moreover, a large number of genes, mutations of which would have cascading, pleiotropic lethal effects on early development, probably exist. Therefore, the high frequency of embryonic lethals is not surprising. Whether or not integration into active genes is favored, it is clear that loci controlling the most complex mechanisms of organogenesis can be affected. Because insertional mutation can lead to cloning of the affected gene, this phenomenon represents a powerful approach to analysis of mammalian development.

Summary

Although it is not proven that transgenes integrate randomly, mutation of genes that effect differentiative processes found only in adult tissues indicates that any gene is susceptible to mutation by insertion. However, because of the size of the mouse genome, it is unlikely that insertional mutations can be reproduced. Because these mutations may be unique and irreplaceable, it is becoming increasingly important that transgenic animals be carefully screened, so that insertions that allow cloning of important genes are not overlooked.

IX. Unexplained Findings

Many aspects of foreign gene behavior in transgenic mice are yet to be fully understood. However, some especially unusual findings deserve particular attention.

First, genes are occasionally unstably expressed even within pedigrees. This bizarre behavior has been well documented in two cases (Palmiter *et al.*, 1983b; Soriano *et al.*, 1986). In the first instance, a transgenic line carrying *pMK*, the mouse MT-1–herpesvirus *tk* construct, was found to express highly variably. Because the insert was short and simple, Southern analysis could be used to demonstrate that variable expression was not due to structural alterations in the transgene between sibs of the pedigree. Soriano *et al.* (1986) found similar results for a recombinant retrovirus carrying a human β-globin gene. Although fewer animals were evaluated, variable expression was clearly observed. In this case, the retroviral insert, because of its specialized integration mechanism, would not be expected to be structurally unstable.

Palmiter *et al.* (1983b) proposed that *pMK* was variably expressed because the gene lacked "organizing sequences," which are normally present in mammalian genes. This mechanism would not explain variable expression in the case of Soriano *et al.* (1986), where the foreign gene was a mammalian gene with its own promoter. Probably, some integration sites are unstable with respect to their permissiveness for foreign gene expression, or are rendered unstable by exogenous DNA integration.

Another unusual finding was made by Shani (1986), who reported amplification of an actin–globin construct after transmission through the germ line in one pedigree. Amplification was sporadic and rapidly stabilized. Therefore, it is possible that the line actually had a distinct integration event in primordial germ cells, which subsequently shrank by internal recombination, a well-documented phenomenon (see, e.g., Palmiter *et al.*, 1982b). However, if amplification actually did take place, it would be a unique observation without explanation.

Also difficult to explain is the finding that expression can disrupt development. Gordon (1986a) noted that several independent lines of transgenic mice carrying an altered mouse dihydrofolate reductase gene manifested a similar pattern of developmental anomalies, suggesting that gene expression was involved. Pinkert *et al.* (1985) noted that three independent lines of transgenic mice carrying MHC class II genes, animals failed to transmit the gene, and one of the animals produced sperm with heads only. Again, a similar defect in independent lines suggests expression as the cause of the anomaly, although the explanation for this effect is unknown.

Summary

As more transgenic mice have been produced and studied, unexplained phenomena have emerged with significant frequency. At present it is difficult to understand these events or even devise strategies for their analysis.

However, lines with such anomalies should be preserved for a time when improved technology and understanding of mammalian development will allow for experimental analysis. These phenomena may provide clues to features of gene regulation of which we are currently ignorant.

X. Future Prospects

It should be evident from this and other reviews that the transgenic mouse system will retain its place as a most powerful tool in mammalian development genetics. In the future, similar experiments to those already conducted will elaborate more crucial information relevant to the most intricate control mechanisms in development. Therefore, the most important future role for the transgenic system is the continuation and broadening of the same kinds of experiments that have already been conducted. However, some new areas are of particular importance.

First, directed integration of foreign DNA is an important goal. While efficient site-directed integration by homologous recombination appears difficult to accomplish from experiments conducted thus far, it remains an important, if elusive, goal to predetermine the integration site of exogenous DNA. Second, the use of antisense constructs to dampen the expression of genes has potential for creating phenocopy mutations and for basic studies of development. Antisense RNA inserted into tissue culture cells is usually not effective unless large molar excesses relative to the sense RNA are present. However, recombinant constructs can be designed to express at higher levels than corresponding endogenous genes by inclusion of more powerful promoter–enhancer elements. Therefore, the transgenic system should be particularly amenable to this experimental strategy. A third important goal is improvement of expression of retroviral vectors. The retrovirus system offers the advantage of a minimally disruptive integration mechanism that leads to insertion of a single copy of the foreign DNA fragment. In addition, the transformation frequency is often high. Fourth, it is important to understand what determines the pattern of expression of recombinant molecules, thereby to predict the tissue distribution of expression. Such knowledge might lead to vector designs that limit foreign gene expression to malignant cells or specific differentiated cell types.

If and when these goals are reached, it may be possible to consider introduction of genes into the human germ line. The obvious application of such technology is treatment of genetic disease. However, it may in the future be feasible to alter lipid metabolism so as to eliminate predisposition to atherosclerosis, or to introduce toxin genes that express only in malignant cells and act as "genetic magic bullets" to eliminate tumors.

These possibilities raise obvious ethical issues that must be confronted, but the potential for gene transfer to improve the genetic fitness of human beings as well as animals cannot be ignored. Whether or not germ-line gene transfer is ever extended to humans, experiments leading to such a decision will provide the deepest insights into one of the most complex and fascinating phenomena of nature—the development and differentiation of the multicellular organism.

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